EFFECT OF DIFFERENT POULTRY PRODUCTION METHODS ON *CAMPYLOBACTER* INCIDENCE AND TRANSMISSION IN THE BROILER MEAT FOOD CHAIN

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MEMORANDUM

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author.

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Maria Camilla Brena

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To my mother

I would not be where I am today without you

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ABSTRACT

Campylobacter is the main cause of human bacterial gastroenteritis worldwide. Within the EU reported cases are rising each year. Epidemiological studies have identified that chicken meat is one of the major sources of human infection. However, it is poorly understood whether differences in chickens' rearing and production methods impact on the contamination levels of *Campylobacter* on chicken meat and therefore the risk of entry into the food chain.

To investigate the role of production system, flocks from diverse broiler commercial production systems with differences in welfare standards, bird type and stocking densities were investigated during the whole rearing period and at slaughter. Caecal samples were collected to estimate the flock prevalence. In order to assess the level of carcass contamination during processing, neck skin samples were collected at different production stages. Breast meat samples were also investigated to estimate the risk that chicken meat poses to human health. The objective was to link the flock *Campylobacter* status to the risk of contamination on the consumer's plate.

All samples were cultured for the presence of *Campylobacter* species. A quantitative method based on ISO 10272-2:2006, was used to determine the level of flock colonisation and *Campylobacter* contamination on broiler carcasses and final products.

Results show that birds reared indoors under higher welfare standards with decreased stocking density with a slower growing breed (Hubbard JA57) had a reduced prevalence of *Campylobacter*, compared to the standard fast growing breed (Ross 308) when grown at the same stocking density. The production system with the higher *Campylobacter* prevalence and the higher *Campylobacter* count in the caecal contents, also reported a greater *Campylobacter* prevalence and counts on carcasses. The bacterial numbers on the final product appeared to be strongly associated with the intestinal colonisation of the slaughter batch. Consequently it is crucial to prevent flock colonisation during the rearing period, to ensure negative flocks are entering into the processing plant. The significance of the aforementioned point was also highlighted by the fact that production stages such as final washing and chilling have little impact in the reduction of contamination of the final product. The high level of contaminated carcasses showed clearly that the chicken meat is putting the UK consumers' health at risk.

An increased incidence of welfare issues, such as pododermatitis and hock lesions, was observed among the production system with the higher level of colonisation, which bring to light a link between *Campylobacter* colonisation and welfare issues. Furthermore, this study emphasised that stressful events such as thinning and transport were followed by an increase in *Campylobacter* prevalence. This highlights the importance of animal health and welfare interactions with *Campylobacter* spp colonisation.

Multi Locus Sequence Typing (MLST) was used to determine how diverse and distinct the genetic *Campylobacter* population structure was among the different commercial production systems investigated. Results showed that all production systems could be potential sources of *Campylobacter* infection in humans with common clonal complexes found. Changes in the prevalence of genotypes associated with the final product compared to those genotypes found in birds arriving from farms were observed. This may reflect the enhanced ability of certain genotypes to resist environmental stressors, such as carcass washing, chilling, chlorine dioxide treatment and oxygen that occur during processing. In this data set, isolates belonging to the ST-257 complex showed a higher tendency to survive in the slaughterhouse environment.

Internal contamination of the breast muscle was also reported in our study, hence posing a further public health threat, as the bacteria contained within the muscle are better able to survive cooking. These studies have demonstrated that this pathogen was highly prevalent among the broiler population investigated. Due to the common extent of this pathogen in food and its impact on human health, it is necessary for the Government bodies, food producers and retailers, to raise consumers' awareness of the *Campylobacter* issue. Particularly the consumers must be made aware of how to manage the risk appropriately during food preparation.

LIST OF ABBREVIATIONS

ACP	Assured Chicken Production
bp	Base pair
CAB	Columbia horse blood agar
CFU	Colony forming unit
CC	Clonal complex relationships
CI	Confidence interval
DEFRA	Department for Environment, Food & Rural Affairs
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
FSA	Food Standards Agency
FBO	Food Business Operator
FCI	Food Chain Information
HACCP	Hazard analysis and critical control points
ISO	International Organization for Standardization
ICC	Interclass correlation coefficient
ml	Milliliter(s)
MRD	Maximum Recovery Diluent
mCCDA	Modified charcoal cefoperazone deoxycholate agar
Min	Minute
MLST	Multilocus sequence typing
NA	Nutrient agar
RSPCA	Royal Society for the Prevention of Cruelty to Animals
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
ppm	parts per million (10^{-6})
QA	Quality Assurance
TAE	Tris–acetate buffer
UV	Ultra-violet light
VBNC	Viable but non-culturable
WHO	World Health Organisation
µl	microliter(s) (10^{-6} l)

CHAPTER 1

INTRODUCTION

1.1. GENUS *CAMPYLOBACTER*

1.1.1. Historical introduction and classification

The genus *Campylobacter* was described for the first time in 1906 during an investigation conducted by the UK government into epizootic abortion in cattle and sheep. The organism was named *Vibrio* and it was identified as an important cause of ovine infertility and abortion (Skirrow 2006). A few years later, an identical organism was isolated from aborting cattle by Theobald Smith et al. and it was named *Vibrio fetus* (Smith and Taylor 1919). The bacterium involved was included in the genus *Vibrio spp* due to its spiral appearance (Moore, Corcoran et al. 2005). It was linked for the first time with enteritis in 1931, when a *Vibrio* was the cause of a winter dysentery characterised by catarrhal inflammation of the small intestine and liver degeneration in cows and calves (Jones and Little 1931; Jones, Orcutt et al. 1931).

The first case of human infection clearly attributed to this pathogen was described in 1957, by Dr. E King (King 1957; King 1962). In 1963 the genus *Campylobacter*, meaning 'curved rod', was differentiated from the *Vibrio* as it was realised that the organism had a different fermentative metabolism and a different DNA base composition compared to the *Vibrio spp* previously identified (Sebald and Veron 1963; Veron and Chatelain 1973).

At present, the family *Campylobacteriaceae* contains 18 species, 6 subspecies and 2 biovars (Humphrey, O'Brien et al. 2007).

1.1.2. Characteristics of the genus Campylobacter

1.1.2.1. Growth and survival characteristics

Members of the genus *Campylobacter* are Gram-negative, most are oxidase-positive (except for *C. gracilis)* and catalase-positive. Cells are slender, curved, S-shaped or spiral shaped (Ng, Sherburne et al. 1985); 0.2-0.9µm wide and 0.2-5.0µm long (Vandamme and De Ley 1991). A single polar flagellum, present at one or both ends of the cell, is responsible for its fast rotating and advancing motility. The bacteria do not form spores and they do not ferment or oxidize carbohydrates (Stahl, Butcher et al. 2012). They obtain energy from amino acids, or tricarboxylic acid cycle intermediates. *Campylobacter* can metabolize mucin (Stahl, Friis et al. 2011) and is well adapted to survive in the mucus film of the caecal and cloacal crypts of the intestinal tract (Lee, O'Rourke et al. 1986).

Campylobacter are microaerophilic bacteria and they have an optimal range of oxygen concentration within 5 to 10%, and 1 to 10% for CO_2 (Bolton and Coates 1983). However, some species can also grow aerobically or anaerobically (Carlone and Lascelles 1982; Chynoweth, Hudson et al. 1998). *Campylobacter* spp. are able to grow within a wide range of pH from 4.9 to 9.0, though the optimal bacterial growth is observed at pH 6.5-7.5 (Alter and Scherer 2006).

Temperature has a significant influence on the survival of *Campylobacter* spp. in the environment and in food. The optimal growth temperature is 42°C, and although they may grow within a wide range of temperatures (32°C to 47°C) (Alter and Scherer 2006), they are very sensitive to high temperature and the bacteria are inactivated relatively easily during the pasteurisation process (Birkhead, Vogt et al. 1988). Although

Campylobacter spp. are unable to grow at temperatures under 30°C and a sudden growth decline near the lower temperature limit is observed, it has been noted that they survive up to 15 times longer at 2°C than at 20°C (Hazeleger, Wouters et al. 1998). At freezing temperatures the ability of *Campylobacter* ssp. to survive decreases rapidly. As result, freezing appears to be an efficient way to reduce the level of *Campylobacter* in chicken meat (Georgsson, Thornorkelsson et al. 2006; Rosenquist, Sommer et al. 2006; Meldrum and Wilson 2007; FSA 2009). However, *Campylobacter* spp. may still be isolated from frozen poultry (Lee, Smith et al. 1998; Sandberg, Hofshagen et al. 2005).

Campylobacter appears to be highly sensitive to environmental factors such as drying conditions and osmotic stress. Many key regulators of bacteria stress defence system found for example in *Salmonella* spp, *Escherichia coli* and *Listeria monocytogenes* are absent in *Campylobacter* (Park 2002; Murphy, Carroll et al. 2006). However, such sensitivity is something of a paradox, given the bacteria's ability to survive outside the host and that it is the leading cause of human bacterial gastroenteritis in the world (Humphrey 2006). During exposure to unfavourable environmental conditions, such as high oxygen concentration, extreme temperatures, low nutrient availability or low osmolality environments, *Campylobacter* can decrease its metabolic activities and undergo morphological transformation from the motile spiral form to a coccoid form. In the adaptation to this degenerative coccoid morphology, *Campylobacter* is believed to enter a viable but non-culturable (VBNC) state (Rollins and Colwell 1986; Keum-Il, Min-Gon et al. 2007). As for other pathogens, it is still not clear whether this coccoid form retains the potential to be revived to a colonisation/infectious form (Ziprin, Droleskey et al. 2003; Oliver 2005).

1.2. CAMPYLOBACTER IN HUMANS

Campylobacter is a zoonotic pathogen and is the main cause of human bacterial gastroenteritis in the world (Humphrey, O'Brien et al. 2007). In Europe, it is estimated that there are approximately nine million cases of human campylobacteriosis per year (EFSA 2011). Since 2005, in the EU, *Campylobacter* has been the most commonly reported gastrointestinal bacterial pathogen in humans. Compared to previous years, in 2009, the number of reported and confirmed human campylobacteriosis cases in the EU increased by 4%. This rise also reflected an overall increase in the campylobacteriosis notification rate in Europe (EFSA 2011). However, it is well recognised that the actual numbers of human campylobacteriosis cases are underestimated as not all cases are reported in the laboratory due to the self-limiting nature of the disease and that it can be associated with mild symptoms (Allos 2001; EFSA 2011; Tam, Rodrigues et al. 2012; Humphrey 2013).

This infection has major economic repercussions on human health care. Indeed there are direct illness costs such as health consultations, laboratory diagnosis, medical treatment or hospitalisation and indirect costs such as loss of work productivity due to sickness, product recalls and legal costs (Roberts, Cumberland et al. 2003; Bogaardt, Mangen et al. 2004; International Consultative Group on Food Irradiation 2009). In the EU the cost of campylobacteriosis to public health systems is estimated to be about €2.4 billion per year (EFSA 2013). The most recent data published by the FSA, indicate that the cost of human campylobacteriosis in the UK is around £900 million per year, which alone represents more than half of the cost for all food-borne infections in the country (£1.5 billion) (FSA 2013).

Certain *Campylobacter* species, e.g. *C. fetus* and *C. jejuni*, are important reproductive tract pathogens in farm animals (leading to abortion and/or infertility issues) (Hedstrom, Sonn et al. 1987; Hum, Hornitzky et al. 2009). Other species, such as *C. helveticus* can cause periodontal diseases. However, the majority of species are implicated in acute enteritis. Within the laboratory confirmed cases of human *Campylobacter* infection, the most often reported pathogenic species is *C. jejuni* accounting for more than 90% of the cases, followed by *C. coli* representing 7% of the infections, with the rest of cases being mainly *C. lari* and *C. fetus* (Gillespie, O'Brien et al. 2002; Moore, Corcoran et al. 2005).

1.2.1. Pathogenesis and virulence factors

Four distinct virulence properties: motility, adherence, invasion, and toxin production have been identified in *Campylobacter* (Walker, Caldwell et al. 1986). Motility is conferred by the single polar long flagellum which can occur at one or both ends of the cell, and it is characterised by darting or corkscrew-like movements. The only exception is *C. gracilis,* which is aflagellate and for this reason is not motile, and *C. howae* which has multiple flagella. The combination of spiral shape and the polar flagella leads to rapid motility that enables the bacteria to reach the attachment site and to penetrate through the mucus layer into the human intestinal epithelial cells. Aflagellate mutants have been used to demonstrate the importance of flagella to colonisation and pathogenesis. Adherence to and invasion of epithelial cells, together with production of toxins such as enterotoxins and cytotoxins, contribute to the enteropathogenicity of these bacteria and they are considered the most important factors for the pathogenesis of human campylobacteriosis (Carvalho, Ruiz-Palacios et al. 2001).

1.2.2. Symptoms of infection

The infectious dose in humans is low and it is estimated to be around 500 bacterial cells (Robinson 1981; Black, Levine et al. 1988; Advisory Committee on the Microbiological Safety of Food 2005).

A typical infection with *Campylobacter* is often indistinguishable from those caused by other enteric pathogens such as *Salmonella*, *Shigella* and *E. coli.* The incubation period is two to five days, and the infection results in an acute self-limiting gastrointestinal illness typically resolved in one week, characterised by mild to severe watery/bloody diarrhoea, fever, nausea, malaise and abdominal pain (Blaser 1997). However, infection in the very young and elderly can be more serious and sequelae following infection can occur, such as Guillain-Barre` syndrome (Allos 1997; Nachamkin, Allos et al. 1998; Allos 2001; Tam, Rodrigues et al. 2003; Tam, Rodrigues et al. 2006), reactive arthritis (Hannu, Mattila et al. 2002; Pope, Krizova et al. 2007), myocarditis (Murphy, Jolly et al. 2013), irritable bowel syndrome and inflammatory bowel disease (Kalischuk and Buret 2010) and may result in hospitalisation. Further research is needed regarding the risk of abortion in pregnant woman infected with *Campylobacter* (Denton and Clarke 1992; Gurgan and Diker 1994; Meyer, Stallmach et al. 1997; Baze and Bernacky 2002).

Mortality rate is poorly defined but low, with deaths normally confined to immuno-compromised patients or those suffering from another severe disease such as bowel cancer (WHO 2000; Allos 2001). No correlations have been found between the severity of gastrointestinal symptoms and the development of sequelae such as Guillain-Barre` syndrome (Allos and Blaser 1995). However, different strains of *Campylobacter* and host

factors were linked to the development of this demyelinating disorder causing acute neuromuscular paralysis (Nachamkin, Allos et al. 1998; Dingle, Van Den Braak et al. 2001).

Human *C. jejuni* and *C. coli* infections do not differ regarding clinical symptoms and duration of illness. However, patients infected with *C. coli* tend to be older than those with *C. jejuni* (Gillespie, O'Brien et al. 2002; Karenlampi, Rautelin et al. 2007).

1.2.3. Diseases pattern and seasonality

Compared to other major food-borne bacteria such as *Salmonella* and *E. coli*, the majority of human campylobacteriosis cases are sporadic and outbreaks are rarely reported (Gormley, Little et al. 2011). Outbreaks when they occur, are mainly associated with raw milk, contaminated water and undercooked chicken liver pâté (Finch and Blake 1985; Altekruse, Stern et al. 1999; Health Protection Agency 2011).

In developing countries, *Campylobacter* gastroenteritis has no seasonal preference (Coker, Isokpehi et al. 2002). By contrast, in developed countries most of the human cases occur from late spring until summer (Kovats, Edwards et al. 2005; Louis, Gillespie et al. 2005). The causes of this seasonal peak are not completely understood. However, links with climatic factors, such as higher temperature, have been reported. In addition, warmer temperatures during summer encourage different eating habits and more outdoor activities, which could increase the exposure to this pathogen (Kovats, Edwards et al. 2005; Tam, Rodrigues et al. 2006; Jore, Viljugrein et al. 2010). Furthermore, the increase in the number of flies observed in summer has been linked to the augmentation of human cases seen during this period (Nichols and Gordon 2005).

1.2.4. Age and sex specific patterns

Campylobacteriosis affects people of all ages (Olson, Ethelberg et al. 2008), although distinct age and sex patterns can be observed in both developed and developing countries. In industrialised countries a higher incidence of disease in the 0-2 year age group is observed (Gillespie, O'Brien et al. 2008). A peak was also seen in the 20-29 year age group, in particular within males, and could be linked to young people moving away from home and starting to cook on their own. During the last few years, an increase of *Campylobacter* cases has also been reported in elderly people (Gillespie, O'Brien et al. 2009; Nichols, Richardson et al. 2012).

In developing countries, infection is still more common in males. It is higher among infants who become immune within the first years, and the disease is rarely observed in older children and adults (Coker and Adefeso 1994; Rao, Naficy et al. 2001; Coker, Isokpehi et al. 2002).

1.2.5. Treatment

Most cases of *Campylobacter* infection are of self-limiting nature and do not require any treatment (Blaser 1997). Nevertheless in case of high fever and prolonged illness with bloody stools, replacement of fluid and electrolytes lost through diarrhoea could be needed.

Antimicrobial therapy is limited by the fact that most patients recover by the time the disease is confirmed by the laboratory test. In the last few decades, the effectiveness of fluoroquinolones has been severely compromised following the emergence of resistant

strains in the early 1990's observed in both developed and developing countries (Allos 2001; EFSA 2008; Ragimbeau, Schneider et al. 2008; Bardoňa, Kolářb et al. 2011). Studies have suggested a strong link between antimicrobial use in food animals and the development of resistance in human isolates in developed countries (Altekruse, Stern et al. 1999; Bodhidatta, Vithayasai et al. 2002; Padungton and Kaneene 2003; Cody, Clarke et al. 2010; Kittl, Kuhnert et al. 2011; Niederer, Kuhnert et al. 2012). This highlights the importance of limiting the use of fluoroquinolones in the treatment of poultry due to the public health associated with the development of resistance in food borne pathogens.

1.2.6. Risk factors associated with human campylobacteriosis

The routes, by which humans are exposed to *Campylobacter* include ingestion of contaminated food and water or direct contact with infected animals or carcasses (Neimann, Engberg et al. 2003). However, there is considerable epidemiological evidence that the most significant risk factor associated with human *Campylobacter* infection is the presence of this organism in chicken (Wagenaar, Mevius et al. 2006; Gormley, Macrae et al. 2008; Sheppard, Dallas et al. 2009). In Europe, different approaches for source attribution of human campylobacteriosis have been taken into consideration (EFSA 2008), including investigations of infrequent outbreaks (Pebody, Ryan et al. 1997), epidemiological studies of sporadic cases (Neimann, Engberg et al. 2003), risk assessment models (Nauta, Jacobs-Reitsma et al. 2007), case control studies (Wingstrand, Neimann et al. 2006) and molecular typing methods (Foley, Lynne et al. 2009). In the last decade, typing methods, such as Multi-locus Sequence Typing (MLST), have shown promising

results for *Campylobacter* source attribution (Dingle, McCarthy et al. 2008; Wilson, Gabriel et al. 2008).

Evidence on the importance of poultry meat as major source of *Campylobacter* in the food chain*,* was provided by a 40% reduction in human campylobacteriosis during the 1999 dioxin crisis in Belgium. The withdrawal of chicken meat and all related poultry products from the supermarkets during the dioxin crisis was the most likely reason for the sudden decline in human *Campylobacter* infections that year in Belgium (Vellinga and Van Loock 2002). During the avian influenza outbreak in the Netherlands in 2003, a reduction in human campylobacteriosis was reported, and again in this case the fall was linked with a reduction of poultry meat consumption (Valkenburgh, van Oosterom et al. 2007). A similar dramatic decline in human campylobacteriosis cases was reported in 2008 in New Zealand: the decline was a consequence of the poultry industry measures that were implemented that year in order to curb the disease (Mullner, Shadbolt et al. 2010). Further indications of the importance of poultry meat as source of *Campylobacter* in the food chain, was given by the Iceland experience. Between the years 1998-2000, the incidence of human campylobacteriosis in this country reached epidemic proportions. The epidemic was almost exclusively traced back to the consumption of fresh chicken and due to the change in the national regulation regarding the retailing of chicken meat: before 1996 only frozen chicken meat was allowed in the national market (Stern, Hiett et al. 2003; Tustin 2010).

As it is now well established that poultry meat is the most significant source of *Campylobacter* in the food chain (Wilson, Gabriel et al. 2008; Mullner, Spencer et al. 2009; Sheppard, Dallas et al. 2009), it can be predicted that a reduction of *Campylobacter*

in chickens will reduce the number of cases in the human population. According to EFSA, handling, preparation and consumption of broiler meat is associated with 20% to 30% of human cases, while 50% to 80% could be attributable to the chicken reservoirs as a whole. Therefore, implementation of *Campylobacter* control measures at the primary production level is expected to have a bigger impact on the reduction of human disease as it would not only reduce the contamination of broiler meat along the food chain but also it would lower the human exposure to the bacteria through pathways other than meat consumption (EFSA 2010d).

1.3. CAMPYLOBACTER IN THE BROILER MEAT FOOD CHAIN

Any strategy to control *Campylobacter* in broiler meat should be based on the strict application of good hygienic practices through all stages of the food chain: in primary production (at farm level); during transportation; at slaughter; and during the final meat processing/packaging. In addition, good hygiene practices need to be followed by retailers, in food outlets, restaurants and in private kitchens, in order to reduce the risk of human infection (Cogan, Bloomfield et al. 1999; de Jong, Verhoeff-Bakkenes et al. 2008; EFSA 2011).

Several interventions at various stages in the white meat food chain have been considered to decrease human campylobacteriosis cases. In some countries, such as Iceland and New Zealand, control programs and intervention studies adopted by the national poultry industry helped to reduce effectively consumer exposure to the pathogen (Stern, Hiett et al. 2003; Mullner, Shadbolt et al. 2010). Prevention of flock colonisation would be ideal to reduce the human risk but this is very difficult to achieve given the current knowledge of this food-borne pathogen. However, reducing the numbers of this pathogen on the final products would still have an important public health benefit (Rosenquist, Nielsen et al. 2003; Nauta, Hill et al. 2009; Swart, Mangen et al. 2013).

1.3.1. Campylobacter in broiler chicken farms

Campylobacter is ubiquitous in nature, and it is found in most animals. However, the avian species are the most common host, probably because of their higher body temperature (42°C), which is ideal for the slightly thermophillic *Campylobacter* species, such as *C. jejuni* and *C. coli* (Skirrow 1977).

Broiler flocks are colonised following exposure to the bacteria from the environment, and the presence of *Campylobacter* in the caeca can be detected after only a few hours (Bull, Allen et al. 2006). It has been reported that the minimum dose of the organism required for chicken colonisation, via the oral route, could be as few as 35 colony forming units (CFU) (Stern 1988), depending on the chicken breed (Stern, Meinersmann et al. 1990; Newell and Fearnley 2003), the age of the birds, the strain and the route of infection (Sahin, Morishita et al. 2002). The principal site of colonisation in birds is the mucus-filled crypts of the intestinal epithelium (mainly caeca and small intestine), and within the crypts, *Campylobacter* cells appear suspended and unattached to the mucosal surface (Beery, Hugdahl et al. 1988).

According to an EFSA survey, the prevalence of *Campylobacter* colonisation in EU broiler flocks detected from the analysis of caecal content samples was 71%. This study highlighted that within the EU countries, *Campylobacter* prevalence varies between 18% to 90%, with a higher prevalence reported in the most southern European countries (de Haan, Kivisto et al. 2010; EFSA 2010a). Further studies have reported lower flock infection rates in Fenno-Scandinavian countries, and this has been linked to enhanced bio-security practices, colder weather, less presence of vectors such as flies, flocks slaughtered at a younger age (33-35 days), no partial depopulation of flocks and other husbandry

parameters such as reduced number of sheds per farm, lower farm density and newer facilities (Barrios, Reiersen et al. 2006).

Many studies have reported that horizontal transmission from environmental sources is the major source of *Campylobacter* infection for broiler flocks (Shanker, Lee et al. 1986; van de Giessen, Mazurier et al. 1992; Jacobs-Reitsma 1995). There has been a major debate on whether vertical transmission is also responsible for the introduction of *Campylobacter* into chicken flocks. One study showed the presence of *Campylobacter* in the reproductive tracts and semen of commercial turkeys and speculated on the potential impact of vertical transmission from the hens to the chicks (Cole, Donoghue et al. 2004). However, the result of *Campylobacter* negative broiler flocks originating from *Campylobacter* positive parent flocks is an indication that vertical transmission is unlikely to occur under commercial conditions (Shanker, Lee et al. 1986; Annan-Prah and Janc 1988; Bull, Allen et al. 2006; Callicott, Friethriksdottir et al. 2006). Therefore, the most likely source of *Campylobacter* introduction into broiler flocks is through horizontal transmission during the rearing period (Jacobs-Reitsma, van de Giessen et al. 1995; Ridley, Morris et al. 2011). However, there is still great uncertainty regarding where *Campylobacter* comes from when it colonises the birds (Conlan, Coward et al. 2007).

The same strains of *C. jejuni* can be seen in the environment around the broiler house (standing water and puddles are sites from which *Campylobacter* can be often recovered, as they protect the bacteria from desiccation), as well as in the chicken flocks (Bull, Allen et al. 2006; Johnsen, Kruse et al. 2006; Messens, Herman et al. 2009; Ridley, Morris et al. 2011). Whilst the latter could indicate a possible common source of contamination that

infects both the environment and the animals, this does not indicate which event (flock infection or environmental contamination) occurs first, and the direction of transmission.

In a poultry farm, the aim of bio-security measures is to protect the birds from exposure to diseases, preventing the entry and the spread of *Campylobacter* and other pathogenic agents into the poultry flock. A conventional, well-maintained poultry farm, with limited access, should be considered bio-secure. However, it is very difficult to obtain and to maintain high levels of bio-security in a poultry farm (Ridley, Morris et al. 2011). It is well documented that human activities (Kazwala, Collins et al. 1990; Newell and Fearnley 2003), flies (Hald, Skovgard et al. 2004; Ekdahl, Normann et al. 2005; Nichols and Gordon 2005; Bahrndorff, Rangstrup-Christensen et al. 2013), wild birds and rodents (Kapperud, Skjerve et al. 1993; Craven, Stern et al. 2000) can be vehicles for the introduction of *Campylobacter* infection into the flocks. Water, as well, could be an important route of transmission into the broiler houses (Pearson, Greenwood et al. 1993; Shane 2000; Herman, Heyndrickx et al. 2003; Saleha 2004). However, drinking water in the commercial farms is normally chlorinated and contamination of the drinking water usually occurs after flock colonisation and not the other way round (Newell and Fearnley 2003). Chlorine is an effective disinfectant against *Campylobacter* and both chlorination and acidification of drinking water have been recommended as control measures to prevent flock colonisation (Kapperud, Skjerve et al. 1993; Pearson, Greenwood et al. 1993).

Once *Campylobacter* has been introduced into a broiler flock, bird to bird transmission is extremely rapid, and the majority (up to 100%) of birds are colonised within only a few days (Shanker, Lee et al. 1990; Berndtson, Danielsson-Tham et al. 1996; Shreeve, Toszeghy et al. 2000; Van Gerwe, Bouma et al. 2005). Indeed, bacteria spread easily in the

chicken farm. Inside the broiler house, communal sources of eating and drinking together with the normal coprophagic activity of broilers, can increase and facilitate the spread of infection (Herman, Heyndrickx et al. 2003; Messens, Herman et al. 2009). In addition this rapid transmission can be a result of the enhanced colonisation potential of the bacteria after *in vivo* passages (Cawthraw, Wassenaar et al. 1996). However, low flock prevalence was described in some UK studies and in the Swedish *Campylobacter* Surveillance Program, which reported the existence of positive flocks with less than 100% of the birds colonised (Allen, Bull et al. 2007; Hansson, Forshell et al. 2007; Hansson, Pudas et al. 2010; Jorgensen, Ellis-Iversen et al. 2011).

1.3.1.1. Pathogenesis of Campylobacter colonisation in chickens

Campylobacter is not an essential component of the normal avian intestinal microbiota since negative flocks occur. After infection, despite the high colonisation rate, the association of *Campylobacter* with poultry does not give any apparent gross pathologic lesions to the chicken host. Similar findings were also observed when young chickens were exposed to the pathogen under experimental conditions (Newell and Fearnley 2003; Dhillon, Shivaprasad et al. 2006). However, the fact that chickens elicit an immune response to this bacteria indicates something other than commensalism (Humphrey 2006). Wieliczko in 1994, reported that as well as *Salmonella*, *Campylobacter* can be responsible for liver diseases in poultry (Wieliczko 1994). Avian hepatitis associated with *C. coli* was also reported in ostriches (Stephens, On et al. 1998). In a more recent study, it was observed that livers of commercial broiler chickens with signs of hepatitis had significantly more *Campylobacter* than those without. However, it was reported that the

presence of *Campylobacter* in the liver alone was not sufficient to cause vibrionic hepatitis (Jennings, Sait et al. 2011).

It is well recognised that the infection outcome is different in humans versus chickens. This is mainly attributed to the ability of the bacteria to invade the intestinal epithelial layer in humans. The characteristics of chickens that appear to inhibit the bacteria from interacting with epithelial cells include the nature and composition of the avian mucus, the higher body temperature, the lower pH of the intestinal tract and the composition of the gut microbiota (Byrne, Clyne et al. 2007). Differential bacterial gene expression in different hosts could also explain the variations in pathogen behaviour in humans and animals (Humphrey, O'Brien et al. 2007).

Campylobacter is not routinely detected in birds younger than 12-21 days old (Berndtson, Danielsson-Tham et al. 1996; Bull, Allen et al. 2006; Johnsen, Kruse et al. 2006), and in commercial broiler flocks the colonisation persists for at least the lifespan of the animals (around 37-42 days for a standard bird in the UK). However, it has been observed that, after 8 weeks, colonisation could decrease in terms of number of bacteria and number of birds colonised which is likely to be associated with the development of an adaptive immunity and changes in the intestinal microflora (Achen, Morishita et al. 1998; Sahin, Naidan et al. 2003; Vandeplas, Dubois et al. 2009).

The early age-related resistance (lag phase), extended against different *Campylobacter* species, is not completely understood. *Campylobacter* specific maternal antibodies (MAB) are common in young chickens and could be involved in this protection: the high level of these antibodies observed during the first weeks, falls at 14 days, reaching minimal levels at 3-4 weeks (Sahin, Zhang et al. 2001; Sahin, Naidan et al. 2003; Vandeplas, Dubois et al.

2009). Also the stage of intestinal development has been hypothesised to be involved in this age resistance, as avian intestinal niches go through physiological change during the first weeks of life (van Der Wielen, Biesterveld et al. 2000). Changes in the microbial flora and competitive caecal microflora (Mead 2002) are also considered in relation to the lag phase, together with management adjustments, such as changes in feed and medication, that occur during the rearing period.

1.3.1.2. Campylobacter seasonality in chicken farms

An important characteristic of *Campylobacter* epidemiology in the chicken is its marked seasonality (Humphrey, O'Brien et al. 2007), with a greater number of flocks colonised during the summer months (July-August) and early autumn (Barrios, Reiersen et al. 2006; Rushton, Humphrey et al. 2009; EFSA 2010b). The shape and timing of this peak varies in different countries, with northern European countries having much sharper summer peaks compared to the southern countries (EFSA 2010a). Seasonality is observed in conventional and also in the more extensive production systems (Vandeplas, Dubois-Dauphin et al. 2010).

This seasonality, which could be explained by greater ventilation of the broiler house that increases the potential contact with the outside environment (Barrios, Reiersen et al. 2006; Guerin, Martin et al. 2007; Jorgensen, Ellis-Iversen et al. 2011) and by a greater exposure to wildlife *Campylobacter* vectors (Chuma, Hashimoto et al. 2000; Hald, Skovgard et al. 2004; Nichols and Gordon 2005), is clearly observed only in non-thinned flocks (FSA 2005; Rushton, Humphrey et al. 2009). Environmental parameters, such as sunshine hours and temperatures, have been also associated with this seasonal

fluctuation (Wallace, Stanley et al. 1997). In addition the stress that the chickens undergo in the summer due to higher temperatures has been linked to an increase in flock colonisation observed during the summer months.

The late spring/early summer peak in human cases precedes the seasonal increase seen in poultry (Newell and Fearnley 2003). Evidence from several countries, such as Denmark, the Netherlands, Norway, Sweden and the UK, suggests that although improved bio-security measures can help to delay the onset of *Campylobacter* colonisation, prevention of flock colonisation cannot be guaranteed with satisfactory bio-security practices during the summer peak (FSA 2005).

1.3.2. Campylobacter at slaughter

In the 'EU baseline survey of *Campylobacter* in broiler carcasses' carried out in 2008, a positive association between the prevalence of *Campylobacter* colonised broilers batches and the frequency of *Campylobacter* contamination of the broiler carcasses at slaughter was observed (EFSA 2010a). *Campylobacter* colonised flocks were 30 times more likely to yield *Campylobacter* contaminated carcasses (EFSA 2011). This correlation was not unexpected, because contamination of the carcasses with *Campylobacter* from their intestines during the automated broiler slaughtering process cannot be avoided completely. Evisceration has been considered a critical step in carcass contamination, as a result of viscera rupture leading to intestinal content release. In addition to direct carcass contamination, intestinal contents contaminate machines (Berndtson, Danielsson-Tham et al. 1996), working surfaces, protective clothing and employee's hands (Jozwiak,

Reichart et al.) increasing the opportunity for cross-contamination of *Campylobacter*-free carcasses (FAO and WHO 2002).

Different studies have shown clearly that cross-contamination occurs during processing. Berrang et al in 2001, reported that carcasses sampled at the end of the processing line, had been contaminated with *Campylobacter* even when the bacteria were not isolated from the chickens on arrival at the abattoir (Berrang, Buhr et al. 2001; Newell, Shreeve et al. 2001; Johnsen, Kruse et al. 2007). Furthermore, it was shown that the subtypes of *Campylobacter* found on the carcasses from colonised birds were not always those which were most prevalent in the gut of the birds (Newell, Shreeve et al. 2001). The large volumes of water used during the processing of the birds could also contribute to the spread of bacteria and can complicate control within the plan (Berndtson, Danielsson-Tham et al. 1996). It is therefore important to ascertain which types of cross-contaminations are occurring during the slaughtering process.

At EU level, the prevalence of *Campylobacter* contaminated broiler carcasses reported during the EU baseline survey, at the end of the slaughtering process, was 75.8% (EFSA 2010b). Within the EU member countries, a tendency was observed for countries having a higher *Campylobacter* prevalence in both slaughter batches and carcasses, to have higher quantitative loads on carcasses (EFSA 2011). A high *Campylobacter* prevalence on the final products, approximately 80%, was also reported in a UK study by Jorgensen et al. (Jorgensen, Bailey et al. 2002). A later nationwide survey undertaken by the FSA between May 2007 and September 2008 showed that the prevalence of *Campylobacter* in chicken meat at retail was 65.2% (FSA 2009).

At slaughter, enhanced design of equipment, prevention of faecal leakage and training of personnel in food hygiene, need to be considered to reduce the contamination in the final products.

1.3.2.1. Campylobacter susceptibly to the disinfectants used in the food industry

One of the 'big issues' in dealing with this pathogen is that *Campylobacter* can enter into the processing plant at very high levels and it is easily spread in the slaughterhouse environment. Even though strict environmental requirements are needed for *Campylobacter* survival and growth (Alter and Scherer 2006), studies show that it is not always easy to remove the organism from the processing plant when it is introduced (Solomon and Hoover 1999).

Overall, this pathogen is considered susceptible to the common disinfectants used in the food industry, especially quaternary ammonium and chlorine compounds (Avrain, Allain et al. 2003). However, Peyrat et al. (2008) detected *Campylobacter* isolates from equipment surfaces in poultry slaughterhouses after the night cleaning operations, suggesting that some genotypes have the ability to survive routine cleaning procedures (Peyrat, Soumet et al. 2008). Slaughterhouse surfaces may vary in sensitivity to bacterial attachment. Attachment for example to the rubber fingers of the de-feathering equipment was found to be significantly lower than attachment to stainless steel and other surfaces (Arnold and Silvers 2000; Reeser, Medler et al. 2007; Nguyen, Turner et al. 2011). The ability for *Campylobacter* to attach to surfaces is facilitated by biofilm formation (Hanning, Jarquin et al. 2008; Nereus and Chen 2009). Within biofilms, bacteria

are protected from the action of the sanitizers, increasing the likelihood of their survival through the food chain and subsequently enhancing the risk of food contamination (Newell, Shreeve et al. 2001; Chmielewski and Frank 2003).

1.3.2.2. Campylobacter and transport of the broilers to the slaughterhouse

During transport, faecal matter accumulates on crates' flooring and can be transferred to the broiler feathers and skin. Furthermore, transport crates can be a point of cross-contamination in the event that a *Campylobacter* negative flock is placed into contaminated crates (Newell, Shreeve et al. 2001; Herman, Heyndrickx et al. 2003; Berrang, Northcutt et al. 2004; Patriarchi, Fox et al. 2011). Depending on the travelling time, birds may stay in the crates for several hours and although this time may not be sufficient for intestinal colonisation, it could be enough to contaminate the external surfaces of the chickens, leading to carcass contamination during processing (Slader, Domingue et al. 2002). Slader et al. (2002) isolated *Campylobacter* from cleaned and disinfected crates: the bacteria present on the crates were probably protected from the disinfectant action by the organic matter. It is well known that the effectiveness of the chlorine compounds is reduced by the presence of organic material (Slader, Domingue et al. 2002; Alter and Scherer 2006).

In addition to the risk of cross-contamination during transport, the failures of the abattoir cleaning and disinfection procedures on transport crates is a concern during the partial depopulation of the flock (thinning), when contaminated crates may enter the broiler house and contaminate the rest of the flock that will be brought to slaughter approximately one week later (flock clearance).

1.3.2.3. Campylobacter and slaughter operations

Within the processing plant, carcasses pass through different steps before becoming final products. Several longitudinal studies have been carried out to estimate which stages lead to an increase or decrease in *Campylobacter* contamination of meat products. It is considered that operations like de-feathering and evisceration cause an increase in *Campylobacter* counts due to leakage of intestinal contents during these processes. Longitudinal studies reviewed by Guerin et al. (2010) showed a general decline in the number of *Campylobacter* towards the end of the processing line: more specifically processes such as scalding, washing and chilling led to a reduction in *Campylobacter* counts on carcasses (Guerin, Sir et al. 2010). Chilling in effect, could be considered a critical control point for the reduction of carcass contamination. However, the chilling process has a limited effect on the number of pathogens on final products if broiler flocks enter into the slaughter process with a high level of contamination (Klein, Reich et al. 2007; Figueroa, Troncoso et al. 2009).

1.3.3. Campylobacter control in the broiler meat food chain

In view of the complex issue in preventing flock colonisation at farm level, and considering that according to the results of the 2008 EU baseline survey, most European countries have *Campylobacter* positive flocks as intake material at slaughter, several options are under investigation to act as corrective actions in the poultry processing line to reduce the number of this pathogen on poultry meat before it reaches the final consumer (SCVPH 1998; Bashor, Curtis et al. 2004; Hugas, Eirini et al. 2007; Cosansu and Ayhan 2010; Loretz, Stephan et al. 2010; Haughton, Lyng et al. 2011; Kudra, Sebranek et

al. 2012). However, with the current control measures that are available during processing and are allowed in the EU, the burden of *Campylobacter* in fresh chicken meat may be reduced but not eliminated completely (SCVPH 1998).

During slaughtering, the detection and removal of faecally contaminated carcasses or their contaminated parts is one way to eliminate *Campylobacter* from the food chain. Physical decontamination, such as freezing, could also be effective (Stern, Hiett et al. 2003; Georgsson, Thornorkelsson et al. 2006; Rosenquist, Sommer et al. 2006). Based on a national surveillance programme, Iceland has legal requirements for freezing (or heat treating) whole batches of broilers, which are shown to be *Campylobacter* positive prior to slaughter. For poultry producers who wish to market fresh poultry meat, this rule is acting as an important incentive to produce *Campylobacter* negative flocks as intake material (Tustin 2010).

It is important to determine at which stages along the food chain, monitoring targets for microbiological criteria and intervention measures could be most effectively implemented. It appears that monitoring at the end of the slaughter line could take into account the effectiveness of any action taken during processing, as well as the farm procedures since all meat products can be traced back to the farm of origin according to the General Food Law Regulation 178/2002, which requires to the Food Business Operators to trace and follow meat products through all stages of production, processing and distribution (FSA 2010). Moreover, the monitoring of meat products at the end of the slaughtering line, can provide quantitative indicators of bacterial load on the final products, which appears to give the most valuable information on consumer exposure to the *Campylobacter* risk (Rosenquist, Nielsen et al. 2003; Swart, Mangen et al. 2013).

1.4. POULTRY INDUSTRY

The chicken meat sector is a global industry, with the big challenge to meet the increased consumer demand for high quality protein and food safety. Intensification of the broiler chicken industry started in the late 1950's, when the use of 'dual purpose' chickens for egg and meat production ceased and new poultry breeds were produced specifically for meat production (Godley 2009). The result of focused breeding programs, led into the international market the selection of a small variety of dominant breeds of broilers which grew two or three times faster, converting food into meat more efficiently (Havenstein, Ferket et al. 1994). Those improvements in breeding programmes, together with developments of production technology and careful control of several management aspects during rearing and processing, were the main factors that allowed the poultry industry to develop into a fully profitable market.

The poultry production systems during those years have changed dramatically from small backyard poultry flocks kept in unspecialised poultry units, to poultry raised in large broiler units at high density. These specialised broiler houses are currently equipped with automated provision of water and feed, controlled temperature, ventilation and lighting to maximise bird growth along with a more intensive production schedule. As a consequence the price of chicken meat has declined and consumption has increased dramatically (Godley 2007).

Worldwide, over 50 billion chickens are reared annually for meat (OIE 2009). In Europe, in 2010, more than 6 billion chickens were slaughtered for meat, with a total of more than 11 million tons of carcass weight produced. After pork, chicken meat is the second most

produced meat in Europe, with an average consumption per head of around 20 kg per annum (AVEC 2011). In the UK, the average annual consumption of chicken meat is just less than 30 kg per person, exceeding any other type of meat (AVEC 2011). According to the National Farmers Union and British Poultry Council, broiler chickens are the most numerous farm animals reared for meat, accounting for approximately one-third of the total UK meat production (Sheppard 2004).

In terms of carbon footprint and land use, chicken meat is the most sustainable meat produced in Europe (AVEC 2011). This fact, together with its dietary and nutritional qualities and the absence of cultural or religious obstacles (AVEC 2011), are the main reasons why even through the recent economic recession, the chicken market was less negatively affected compared to other types of meat production (USDA 2011).

1.4.1. Structure of the UK poultry industry

Worldwide the meat poultry industry can be separated into an independent international primary breeding sector and a more national or regional production sector (Figure 1.1). The primary breeding sector consists of a small number of genetic companies producing a limited number of broiler breeds suitable for the different production sectors of the poultry market all over the world. These are: Cobb-Vantress (Cobb , Avian and Sasso brands) Aviagen (Ross, Arbour Acres and Lohmann Meat strains) and Hubbard (EFSA 2010c; de Jong, Berg et al. 2012).

In the UK, the two largest multi-national breeding companies (Aviagen and Cobb-Vantress) supply over ninety per cent of the broiler stock in the country, and only one of

them (Aviagen) has pedigree stock in the country (DEFRA 2006). A higher level of bio-security is maintained in all pedigree farms, as this is the first important step to keep pathogens out of the food chain. Eggs are hatched in a pedigree hatchery and their progeny is sent onto the great grandparent and grandparent generations who produce parent stocks that are passed then into the production sector.

To achieve profit and the large scale of production requested (International Consultative Group on Food Irradiation 2009), the production sector in the UK is concentrated primarily in few very large integrated poultry companies that own or control parent flocks, growing farms, feed mills and processing meat plants. In 2002, the four largest UK poultry companies covered 70% of all the UK poultry production (Sheppard 2004).

Parent stocks are purchased at one day old from a primary breeding company, and they are placed on rearing farms, where males and females are reared separately from one day old until the mating period (which occurs between 21-24 weeks) to improve flock performance. Animals are then moved into production farms, where eggs are collected on a weekly basis; thereafter the eggs are sent to the hatcheries. On the day the eggs are hatched, the chicks are sent directly to the broiler growing farms where they will remain until they go to slaughter.

There were approximately two and half thousand broiler farms registered in the UK in 2010, keeping more than 120 million broilers at any one time over the whole country (Crane, Davenport et al. 2012). The vast majority of farms are either owned by the integrated companies or are tied to the integrator by long term contracts that usually involve the supply of day old chicks, feed and a strong supervision of farm management practices. In these farms, birds are reared on floors covered with litter that is removed at

the end of each production cycle. Generally each farm produces between 5 and 8 flocks per year. Each flock is sent to slaughter split into one or more slaughtering batches. The depopulation process can be carried out over different days depending on the flock size and the different market weight demand.

In Europe, the UK has the highest slaughtered broiler population, followed by France, Spain and Poland (EFSA 2010a). In 2012 almost 900 million chickens were slaughtered in the UK, resulting in around 1.3 million tonnes of chicken meat produced for the poultry market (DEFRA 2013)

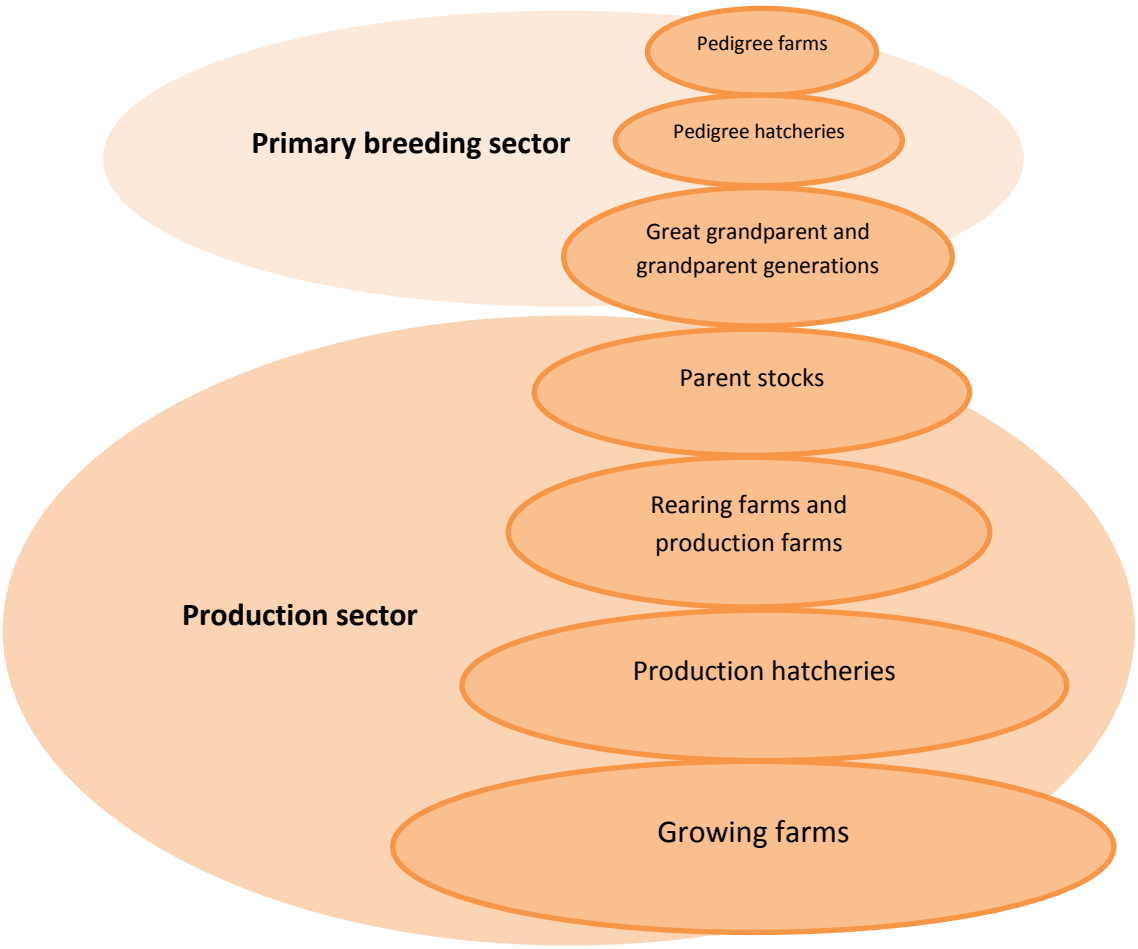

Figure 1.1: Structure of the UK poultry industry

1.4.2. Broiler production and animal welfare

In the last 50 years, in order to keep the chicken prices at retail as low as possible, the poultry sector has focused its objectives on increasing productivity rather than in animal welfare. As a result the modern phenomenon of 'cheap meat' comes at cost to the welfare of the animals involved (Rauwa, Kanisb et al. 1998; Scientific Committee on Animal Health and Animal Welfare 2000; EFSA 2010c).

Characteristics of commercial importance such as fertility, daily growth rate, feed conversion ratio, carcass conformity and meat yield are continually improved by the breeding companies, and products are selected to ensure they are able to perform to the highest standards in a wide variety of environments (Arthur and Albers 2003; Aviagen 2012). While the industry claims that other traits such as low frequency of leg disorders or resistance to pathogens are included in the selection programmes, others argue that the importance given to such traits is often low and up to now has not improved the bird welfare (Scientific Committee on Animal Health and Animal Welfare 2000).

Several studies showed that the current high growth rate of commercial broilers that allows standard birds to reach 1.5 kg of body weight in 30 days, compared to 120 days needed in the 1950s (Havenstein, Ferket et al. 2003; EFSA 2010c) has resulted in a number of physical and metabolic disorders in the animals (Rauwa, Kanisb et al. 1998; RSPCA 2011). This high growth rate, combined with management practices that do not provide the chickens with an environment that meets their needs (for example a stimulating environment that allows the animals to exhibit their natural behaviour) is detrimental to their welfare. This compromised animal welfare is leading to higher flock mortality rates, a higher proportion of leg problems, heart failures, sudden death

syndrome, ascites and behaviour problems such as cannibalism when they are only a few weeks old (Kestin, Knowles et al. ; Gardiner, Hunt et al. 1988; Kestin, Knowles et al. 1992; Julian 1998; Sanotraa, Lunda et al. 2001; Bokkers and Koene 2003; Bessei 2006; Estevez 2007; Knowles, Kestin et al. 2008; EFSA 2010c). Other welfare problems, such as contact dermatitis, are also related to management quality: poor control of litter humidity being one example. Floor litter moisture can be affected by drinker design, high stocking density, diet, ventilation and gut health (Ekstrand, Algers et al. 1997). Poor control of litter humidity can result in breast dermatitis, and other lesions such as hock marks and pododermatitis that have been correlated with lameness (Martland 1985; Mayne, Else et al. 2007; Allain, Mirabito et al. 2009). Evidence suggests that such lesions can cause pain to the animals and may also act as a route for bacterial infections (Martland 1985; McGeown, Danbury et al. 1999; RSPCA 2011).

However, over the recent years animal welfare in poultry production has become an increasingly important concern in Europe and more attention is being given to its regulation (Van Horne and Achterbosch 2008). In May 2007, the European Commission agreed a new Directive, EC/2007/43, covering for the first time across Europe the welfare of broilers. The main provision of the Directive is the regulation of stocking density by setting a maximum density of 33 kg per m^2. The Directive allows for stocking density to be increased to a maximum of 39 kg per m^2 under certain conditions such as good ventilation and temperature control systems inside the broilers houses. Moreover, under exceptionally high welfare conditions, for example low flock mortality rates, the density could be further increased by 3 kg per m^2. Before the implementation of this EU Directive, the average density used by the poultry industry in the EU was 40 kg per m^2. In France the

stocking density was up to 42 kg per m² and in Belgium up to 45 kg per m² (Audran 2010). This Directive also covers lighting, litter, feeding, ventilation and staff training requirements. In December 2010, the Regulations to implement the Council Directive 2007/43/EC on chicken grown for human consumption came into force in the UK, governing bird welfare and bringing some improvements in the intensive commercial farming systems. However, not all the animal welfare indicators were taken into account, as according to this regulation assessment of broiler welfare is focused mainly on stocking density and mortality rate.

Nowadays the search for good welfare conditions and for more 'natural' products (organic systems) is a global tendency in animal production. Producers and retailers have been encouraging consumers to spend more on chicken products certified by organic bodies and welfare friendly schemes such as Label Rouge in France and Freedom Food in the UK. These schemes ensure animal welfare standards above the legislation requirements, providing the animals with more space, daylight, natural ventilation, stimulating environment and access to the outdoors.

According to the British Poultry Council, the indoor standard production (the UK conventional broiler commercial system) accounts for 81% of the UK broiler market, with the rest of the market being filled by higher welfare indoor production systems or other less conventional systems (Crane, Davenport et al. 2012) (Figure 1.2). Free range broiler production in the UK is increasing substantially and supplies 6% of the chicken meat in the supermarket. Organic production as well is one of the most expanding sectors in the food poultry industry in many European countries, with its major limitation being the higher

prices of the products (Eurogroup for animals 2010). In the UK, organic poultry products are less than two per cent of the total market (Crane, Davenport et al. 2012) (Figure 1.2).

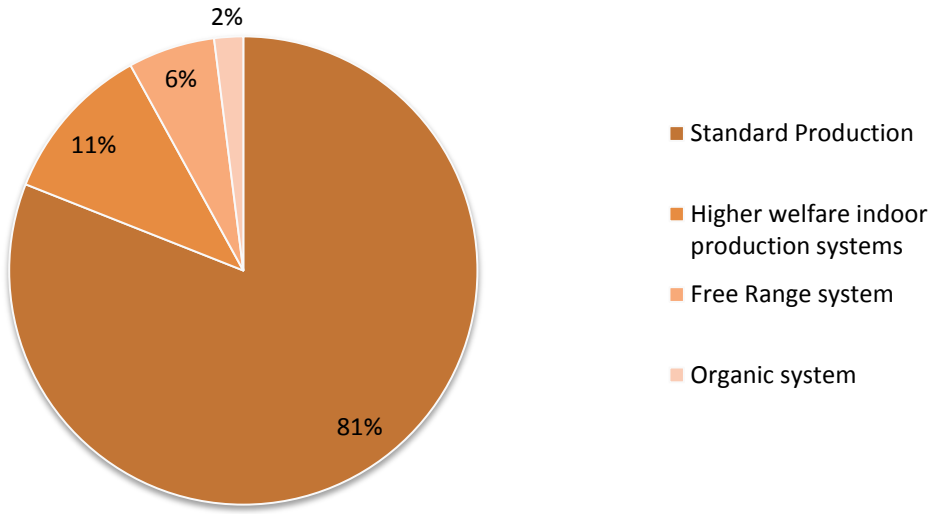

Figure 1.2: UK broiler market by commercial poultry production system

1.4.2.1. Commercial broiler systems: welfare, stress, Campylobacter and public health

Several studies showed that animals reared intensively in poor welfare conditions, are more susceptible to infections due to the immunosuppression caused by stress (Dohmsa and Metzb 1991; Leitner and Heller 1992; Mashaly, Hendricks et al. 2004; Shini, Huff et al. 2010). Furthermore, links between stress levels and pathogenesis of infectious diseases have been reported (Peterson, Chao et al. 1991; Callaway, Morrow et al. 2006; Everest 2007; Burkholder, Thompson et al. 2008; Rostagno 2009). Stress has been shown to be a factor in decreasing the ability to cope effectively with bacterial infection, suggesting that birds with high stress levels could be more susceptible to infection with *Campylobacter* and other pathogens (Leitner and Heller 1992; Rosales 1994). Acute stress suffered for example by birds during catching and transport, has been associated with an increase in

the rate of faecal shedding of *Campylobacter* (Stern, Clavero et al. 1995; Byrd, Corrier et al. 1998; Whyte, Collins et al. 2001).

It is clear that intensive management and the environment affect bird welfare and their resistance to infections, therefore increasing the public health risk from some zoonotic diseases (Rauwa, Kanisb et al. 1998; Humphrey 2013). However, in infections with, for example, *Campylobacter*, the potential of the farm environment to act as a source of the pathogen for the poultry flock, suggests that in extensive production systems where animals have access to the outdoors, a higher *Campylobacter* prevalence is reported (Engvall 2001; Heuer, Pedersen et al. 2001; Luangtongkum, Morishita et al. 2006; John Tang Yew Huat 2010).

The increased concern of consumers about animal welfare (EFSA 2010e), with the rising popularity of extensive production systems and a growing demand for meat produced from animals reared under higher welfare, raises the question of whether the animals' benefits of growing under higher welfare production systems are compatible with food safety and public health (Engvall 2001; Humphrey 2006). It is important to evaluate if the benefits of keeping animals under higher welfare conditions, reducing the negative impact of stress on susceptibility to infection, are balancing the public health risk with the potentially greater exposure to pathogens among the more extensively reared birds (Figure 1.3).

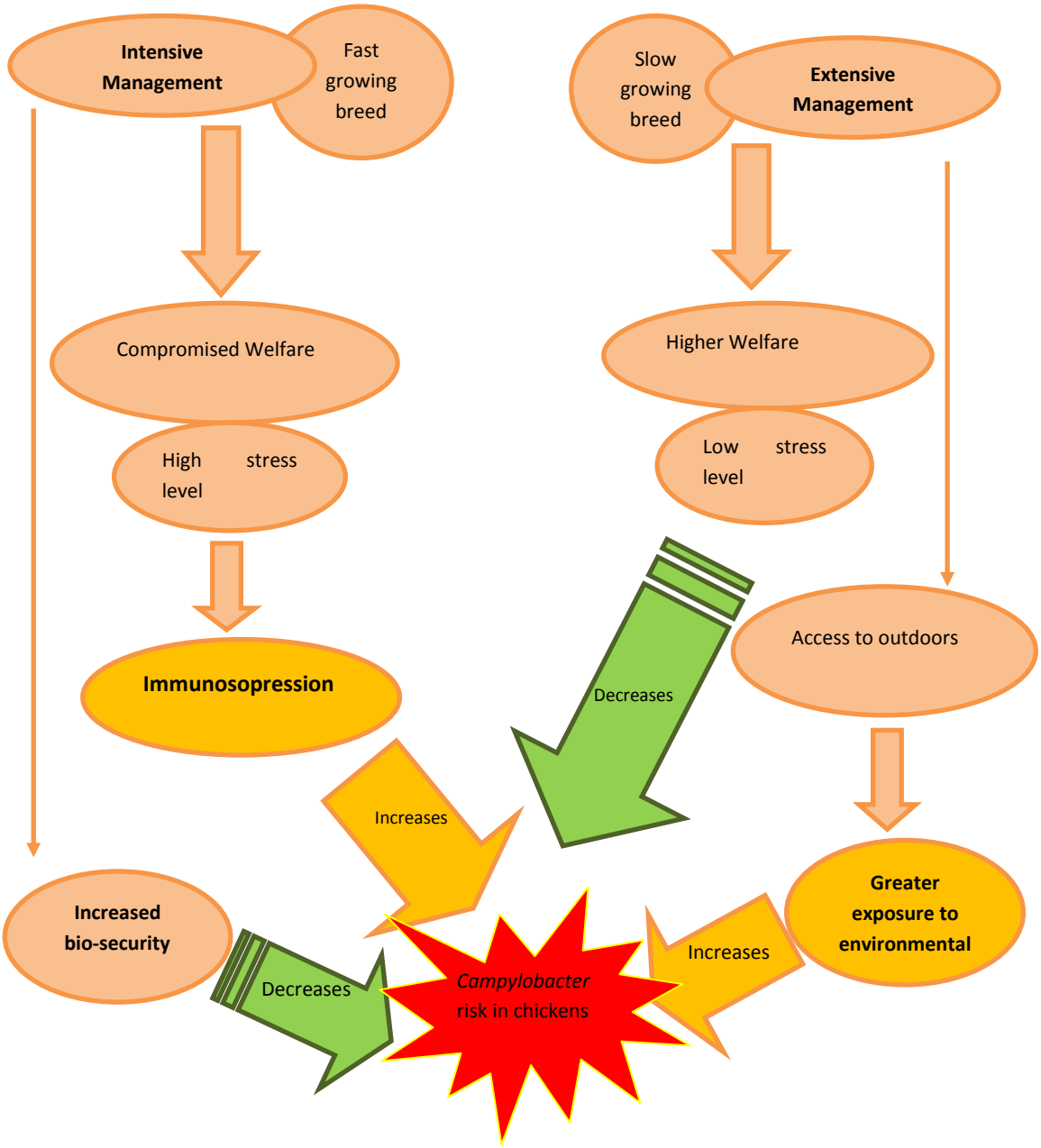

Figure 1.3: Contradictory effects of production systems on public health risk

1.5. AIMS AND OBJECTIVES OF THIS THESIS

The overall aim of this thesis was to investigate the effect of different poultry production methods on *Campylobacter* incidence and transmission in the broiler meat food chain.

More specifically the main aims were to:

- Estimate the differences in *Campylobacter* colonisation in four different commercial broiler production systems;
- Investigate the role of commercial production systems on *Campylobacter* prevalence;
- Quantify the *Campylobacter* colonisation levels in the UK poultry population among different commercial production system;
- Investigate the association of different welfare parameters on *Campylobacter* prevalence;
- Quantify carcass contamination rates in UK broiler population and to investigate the factors that might influence carcass contamination during processing;
- Determine the diversity and population structure of *Campylobacter* among the different commercial production systems.

CHAPTER 2

DIFFERENT COMMERCIAL POULTRY PRODUCTION METHODS AND CAMPYLOBACTER PREVALENCE AT SLAUGHTER

2.1. INTRODUCTION

Campylobacter is a zoonotic bacterium and one of the main causes of human gastroenteritis in the world. In the UK and the EU reported cases of human campylobacteriosis are rising each year (EFSA 2012; HPA 2012; Tam, Rodrigues et al. 2012). Through epidemiological studies, it is well recognised that chicken meat is one of the major sources of human infection (Sheppard, Dallas et al. 2009). However, there are few data on whether the contamination levels of *Campylobacter* on chicken meat and therefore risk of entry into the food chain, varies with chickens reared under different commercial production methods.

According to DEFRA, in 2012, 873.81 million commercial broiler chicks were placed in UK farms, resulting in more than 1.3 million tonnes of chicken meat produced in the country (DEFRA 2013). Even if consumers are becoming more aware of production methods used for animals kept for meat production and would be prepared to pay more to increase the 'happiness' of the animals they eat, many people still do not know about the welfare problems affecting chickens reared for meat production (Eurogroup for animals 2010; Silva, Nääs et al. 2011; Bennett 2012).

To achieve the large market demand and to produce ever cheaper meat, commercial broilers in the last fifty years have been intensively selected for faster growth and for traits that are desirable for meat production which have resulted in major changes in the anatomy and physiology of broilers. The result of these narrowly focused breeding programmes has been a broiler chicken which grows twice or three times faster than traditional breeds and which converts its food into meat in a much more efficient way

(Havenstein, Ferket et al. 1994; EFSA 2010c). Consequently the price of chicken meat has declined and consumption has risen dramatically (Godley and Williams 2007).

The negative consequences of these fast growth rates, combined with management practices focused to increase the production, are the enormous costs to the welfare of the animals involved (Rauwa, Kanisb et al. 1998). Currently the majority of broilers are raised under intensive systems in closed buildings, in which the temperature, lighting, ventilation and nutrition are controlled to ensure the highest and quickest growth rate possible. Birds reach slaughter weight at 5-7 weeks of age. In some cases, animals are overcrowded and the lack of space reduces their ability to move around and extends contact with the soiled litter, leading to ammonia burns to their feet, legs and breasts (Estevez 2007). Inside the poultry house, the light is kept low to discourage birds' activity, and it is often left on continuous to encourage the birds to eat constantly to increase body weight as quickly as possible, with the negative effect of not providing adequate rest to the animals.

In the last few years, consumers have become more conscious of how their food is produced, and they are more sensitive to the animals' production methods and their welfare (Van Horne and Achterbosch 2008; EFSA 2010e). This increasing concern of consumers for animal welfare means that alternative production systems that ensure animal welfare standards above the legislation requirements, are being adopted by the poultry producers. Slower growing breeds are being introduced, combined with management practices that give the animals the opportunity to express their natural behaviour such as access to the outdoors, perching and foraging.

These more extensive production systems, provide the consumers with alternative choices over the methods by which their food is produced, and satisfy consumer' concerns mainly associated with animal welfare and environmental sustainability. However, potential benefits or detrimental effects on food safety and public health need to be taken into consideration to assess the development of this growing market for meat produced from animals reared under higher welfare standards (Engvall 2001).

With pathogens such as *Campylobacter,* where a higher prevalence has been reported in the more extensive systems (Engvall 2001; Heuer, Pedersen et al. 2001), it is important to evaluate if the benefits from growing under higher welfare production systems are compatible with food safety and public health. Furthermore it is necessary to establish whether chickens reared under different commercial production systems are posing the same health risk to humans (Humphrey 2006).

In the last years, despite the important progress that has been made in the understanding of the epidemiology of *Campylobacter* in chicken flocks, relatively little is known about the impact of poultry production systems on *Campylobacter* incidence. Since colonised poultry flocks are considered an important source of *Campylobacter* infection in humans and the demand for extensive systems is increasing in the UK, we conducted a cross-sectional study at slaughter to determine the impact of the production system on *Campylobacter* colonisation and transmission through the white meat food chain. A quantitative approach has been used in this study, as *Campylobacter* remains a concern even at low level (Nauta, Jacobs-Reitsma et al. 2007).

More specifically the main goals of this study were to:

- Estimate the differences of *Campylobacter* colonisation in four different commercial broiler production systems;
- Quantify the *Campylobacter* colonisation levels in the UK broiler chickens among four different commercial production systems;
- Investigate the association of different farms and welfare parameters on *Campylobacter* incidence and prevalence;
- Quantify carcass contamination rates among the different production systems;
- And finally to estimate the risk factors associated with the presence of *Campylobacter* at slaughter that influence carcass contamination.

2.2. MATERIAL AND METHODS

2.2.1. Study design

The role of the production system on *Campylobacter* prevalence was investigated at slaughter in four different commercial production systems. The production systems were chosen within one integrated UK poultry producer and they differed mainly in breed used, stocking density and access to the outdoors. Other welfare parameters, such as lighting requirement, environmental enrichment, flock size and growth rate were also distinct among the different production systems.

Three high volume slaughterhouses (A, B and C) were involved in this project, and they were selected according to the broiler production system processed and their proximity to the laboratory facilities. In each plant, the days of sampling were planned ad hoc according to the weekly workload. The batch to be sampled was chosen ad hoc in the processing plant on the day of sampling.

At slaughter, from each selected batch, 10 caecal samples were collected at evisceration to estimate the *Campylobacter* colonisation. Caeca were chosen as they are the main site of *Campylobacter* colonisation in the chickens (Beery, Hugdahl et al. 1988). In order to assess carcass contamination during processing, 10 neck skin samples were collected at the end of the slaughter line, after the air-chilling process and before any further processing.

The calculation of the number of farms to be sampled was based on the results from a pilot study (first 10 farms, not including any of the Free Range ones). The variances were

calculated using random effects linear regression with farm as the cluster factor and farm type as the dependent variable. Subsequently, the Interclass Correlation Coefficient (ICC) was used to calculate the number of clusters that should be included in the study using the following formula (Kirkwood and Sterne 2003):

$$\text{number of clusters} = \frac{n}{n'}[1 + \ n' - 1 \ x \ \text{ICC}]$$

Where n = uncorrected total sample size and n' = average cluster size.

The Interclass Correlation Coefficient (ICC) was calculated by the following formula (Kirkwood and Sterne 2003):

$$\text{ICC} = \frac{\sigma_u^2}{\sigma_u^2 + \sigma_e^2}$$

Where σ_u: between cluster variance and σ_e: within cluster variance.

In order to establish if the results of the study were satisfying the desired power of 80% we compared cluster sizes that would have been required for each farm type with those used during the study. The required numbers of clusters were calculated using the following formula (Hayes and Bennett 1999):

$$c = 1 + \ Z_{\alpha\ 2} + Z_{\beta}\ ^2 \ \frac{\frac{(\sigma_0^2 + \sigma_1^2)}{n} + k^2(\mu_0^2 + \mu_1^2)}{(\mu_0 - \mu_1)^2}$$

Where: *$Z_{\alpha/2}$ and Z_{β} are standard normal distribution values corresponding to upper tail probabilities of $\alpha/2$ and β respectively. σ_0 and σ_1= standard deviations of the log-mean Campylobacter counts for the different farm types. k = the coefficient of variation of true means.*

The sample was calculated to provide a power 80% of obtaining a significant difference at $p<0.05$ for a difference (μ_0-μ_1) in log-mean *Campylobacter* counts.

The required cluster numbers and those used in this study are presented in the following table (Table 2.1):

Farm types compared	Required number of clusters (farms)	Actual number of clusters (farms)
Standard v Freedom Food indoors	14.743	16/8
Standard v Improved welfare	5.166	16/9
Standard v Freedom Food Free Range	29.39	16/14
Freedom Food Indoors v Improved Welfare	2.077	8/9
Freedom Food Indoors v Freedom Food Free Range	4.984	8/14
Improved Welfare v Freedom Food Free Range	14.510	9/14

Table 2.1: Cluster numbers required and actual number of clusters used in this study (α:0.05; β:80%)

2.2.2. Broiler production systems

Within the UK poultry industry, different welfare systems among the commercial broiler production are determined according to stocking density, access to outside areas, growth rate, lighting requirement and other welfare parameters. The four commercial broiler production systems investigated in this study were: Standard production system (ST), RSPCA Freedom Food system (FI), Improved Welfare production system (IW) all reared indoor, and RSPCA Freedom Food system (FR) reared free range.

2.2.2.1. Standard production system (Ross 308, 38 kg/m²) (ST)

All Standard chickens included in this study were reared according to the standards developed by the UK chicken industry's own assurance scheme: Assured Chicken Production (ACP) (RTFA 2011). According to this scheme, broilers are raised in large groups, in insulated buildings with controlled environments including artificial light. In this closed environment, broilers are kept on litter with automated provision of high energy feed and water. A commercial breed, Ross 308, selected for rapid growth and able to perform consistently in the broiler house is used (Aviagen 2012).

In all Standard farms the birds are sexed, through the technique of feather sexing when they are one day old. Males and females are kept separated inside the broiler house. The purpose of this procedure is that both sexes can be managed more efficiently with regard to feeding and stocking density, as well as improving the uniformity of the flock at slaughter. Depending on the final weight needed to be reached for business purposes, female or male chickens left the farm in consecutive catch lots, carried out also on successive days.

2.2.2.2. RSPCA Freedom Food indoor (Hubbard JA57, 30 kg/m²) (FI)

Established in 1994, the Freedom Food scheme is the Royal Society for the Prevention of Cruelty to Animals (RSPCA)'s farm assurance and food labelling scheme (RSPCA 2011). The aims of this scheme are to improve and to promote higher welfare standards among animals farmed for food, and to offer consumers a welfare choice when buying their meat products. It is the only UK farm assurance scheme focused exclusively on improving the welfare of farm animals reared for food (RSPCA 2011). In 2011 in the UK, 75 million farm

animals (8% of the UK's population) were reared under this scheme (McNair, Spelman et al. 2013). The scheme assesses farms, hauliers and abattoirs against the RSPCA's welfare standards, and only if the assessment is successful can their products be labelled as Freedom Food.

In these farms, flock sizes generally do not exceed 30,000 birds, and the animals are kept in houses where the stocking density is around 20% lower than standard systems. The recommendation of the EU Scientific Committee on Animal Health and Animal Welfare that "above 30kg/m² (up to 15 chickens/m²) welfare problems are likely to emerge regardless of indoor climate control capacity" was followed by the RSPCA's Freedom Food scheme, which sets a maximum density of 30kg/m² in all its broilers farms.

In Freedom Food indoor farms, chickens are raised in a more stimulating environment. Perches, nest boxes, straw bales, pecking blocks and reflective surfaces are available inside the broiler house, which encourages birds to be more active, reducing leg and skin problems. To avoid broilers being reared under an almost continuous lighting regime, lighting requirements are also regulated by the RSPCA's standards which set a minimum of 8 hours continuous light and a minimum of 6-12 hours continuous darkness over a 24 hour period. Keeping the lights on, in fact, encourages the birds to feed for longer periods, which maximises their growth rate but affects their welfare by preventing animals from having an adequate dark period for resting. The maximum growth rate of the birds also is set by the scheme, and it must not be greater than 45 grams per day. As a guide, birds should reach a body weight of around 2.2kg by the time they are 49 days old (RSPCA 2011). For this purpose, animals are fed with low energy/protein feeds and a

slower growth broiler breed, Hubbard JA57 is used (Hubbard 2012). Chickens belonging to this farm assurance scheme are not separated by sex during their whole life.

2.2.2.3. Improved Welfare production system (Ross 308, 30 kg/m²) (IW)

The Improved Welfare production scheme has been commissioned by a UK food retailer, which only approves farm producers that can raise poultry under specific welfare requirements. Within this system, chickens are subjected to higher welfare conditions during the rearing period, compared to birds raised only under ACP standards. All farms have windows, which provide birds with natural day light. Therefore creating a more natural and stimulating environment, with an increased resting time. Each bird has also more space than a Standard chicken. Although these higher welfare standards address some important wellbeing concerns when animals are kept in the farms, this scheme does not meet the requirement for slower growing breeds as specified within the RSPCA's standards. The breed, Ross 308 (Aviagen 2012), used in this commercial production system, is the same as the one used in the Standard production system.

2.2.2.4. RSPCA Freedom Food free range (Hubbard JA57, 30 kg/m²) (FR)

Free range chickens belonging to this production system, are included in the Freedom Food RSPCA's farm assurance and food labelling scheme (RSPCA 2011). The same slower growing broiler breed used in the indoor system, Hubbard JA57, is used in all free range farms belonging to this scheme (Hubbard 2012). All flocks have access to the outdoors. However all free range flocks are placed firstly in indoor containment before free range release. By regulation, these birds must have access to the outdoor range by the time

they are 28 days old, with a maximum of 1000 birds per hectare (RSPCA 2011). The Freedom Food labelling scheme sets the flock size in the free range system to a maximum of 15,000 birds per flock (RSPCA 2011). This is done to encourage the birds to use the outdoor range and to improve disease management, as free range birds have a greater bio-security risk.

	Standard System (ST)	**Freedom Food Indoor (FI)**	**Improved Welfare System (IW)**	**Freedom Food Free Range (FR)**
Standards	ACP (Red Tractor)	RSPCA	Supermarket standard	RSPCA
Breed	Ross 308	Hubbard JA57	Ross 308	Hubbard JA57
Sex	Sexed	As hatched	Sexed and as hatched	As hatched
Stocking density (kg/m^2)	38	30	30	30
Housing & environment	Standard	Standard with windows	Standard with windows	Standard with windows and range
Environmental enrichment	N/A	Straw bales, perches and pecking objects	Straw bales, perches and pecking objects	Straw bales, perches and pecking objects
Range requirement	N/A	N/A	N/A	From 28 days old max, 1000 birds per hectare max
Thinning requirement	N/A	Full depopulation (Minimum at 49 days)	N/A	Full depopulation (Minimum at 49 days)
Stunning requirement	No set standards, however birds must be rendered unconscious	Stun to kill	Stun to kill	Stun to kill

Table 2.2: Features summary for the 4 production systems investigated

2.2.3. Data and sample collection

Ten caecal and 10 neck skin samples were collected from each batch at slaughter over a two-year period from 23rd August 2010 until 07th July 2012. The 'slaughter batch', was defined as a group of chickens from the same flock delivered to the same slaughterhouse in the same vehicle, whereas the "flock" was defined as a group of chickens placed in the farm at the same time and raised in the same broiler house. Only one batch for each flock was sampled. No batch information, including flock *Campylobacter* status, was available before the sampling.

Caecal samples were collected at the time of carcass evisceration by manual traction at the junction with the intestine. Neck skin samples were collected at the end of the slaughtering line, at the exit of the air-chilling process. Only intact caeca were collected, and neck skin samples were collected from carcasses free from any visible contamination. After collection, samples were kept individually into a sterile bag, under refrigerated conditions until analysis. Samples were tested within 2-48 hours depending on the location of the slaughterhouses.

On the day of sampling, flock information was collected from the Food Chain Information (FCI) made available for each batch sampled by the Food Business Operator (FBO) (Appendix 1). The FCI, produced by the rearing farm, provided information on flock size, breed, stocking density, flock mortality at different ages, disease history, diseases diagnosed and medications prescribed. The FCI is an official document consulted by the Official Control Body in the plant to take decisions about the flock, and it is used to determine specific inspection procedures.

During the sampling, batch welfare parameters such as pododermatitis, hock marks, total and partial rejection of products declared unfit to enter into the food chain were assessed and figures recorded. Total carcass rejections included rejections of whole carcasses due to: dead on arrival, cull, ascites, hepatitis, perihepatitis, pericarditis, emaciation, fever, bruising and skin lesions. Factory rejections, such as uncut, over-scald and machine damage, were not included into the analysis. Partial rejections, due to hepatitis and pericarditis when only localised lesions were observed at post mortem and only the chicken offal packet was rejected, were also recorded. Processing data such as batch catching time, time of arrival on site, time of processing, killing order, line speed, temperature of the water in the scald tank, temperature inside the air-chiller, time of air-chilling process, carcasses temperature at the exit of the air-chiller and concentration of chlorine dioxide in the water supply were recorded. Furthermore, slaughter operations, hygiene standards and visible carcass contamination were evaluated during the processing. A questionnaire, including all flock, batch and slaughterhouse information, was completed for each slaughter batch sampled (Appendix 2). Table 2.3 summarises at which level data were collected.

Data collected at farm level	Data collected at flock level	Data collected at batch level	Data collected on individual birds
Breed	Stocking density	Age at slaughter	*Campylobacter* count
Feed Mill	House mortality	Weight at slaughter	
Flock size	Disease history	Rejection at slaughter	
Number of houses	Diseases diagnosed	Pododermatitis	
	Medications prescribed	Hock burns	

Table 2.3: Level at which data were collected

2.2.4. Quantification techniques

All the samples were processed, individually, for qualitative/quantitative detection of *Campylobacter*. At the arrival in the laboratory, neck skin samples were always processed first. Previous studies showed that *Campylobacter* numbers on chicken carcasses remain largely unchanged over 7 days at refrigeration temperature (Jorgensen, Bailey et al. 2002). Caecal samples were kept chilled and left intact until the time of testing as a better *Campylobacter* survival rate was reported in chilled caeca samples. Studies have shown that *Campylobacter* counts remained unaffected up to 80-96 hours after collection (Bull, Thomas et al. 2008; Rodgers, Clifton-Hadley et al. 2010). For both samples (neck skin and caecal sample), the enumeration methods were based on the International Organisation for Standardisation (ISO) methods 10272-2:2006 (ISO 2006).

2.2.4.1. Enumeration of Campylobacter in neck skin samples

A test portion of 15 grams of neck skin was transferred to 45ml of Maximum Recovery Diluent (MRD) (Lab M, Lab 103), and the mixture (1 in 4) was treated in a stomacher (Micro-Biomaster, Seward Stomacher 80, Lab System) for one minute at normal speed. Ninety ml of MRD were added subsequently in order to adjust at a 1 in 10 solution. The decision to add the solution in two steps was due to the small size of the stomacher available. From the initial suspension, serial dilutions were prepared adding 0.5ml of sample to 4.5ml of MRD. Dilutions 10^{-1} to 10^{-3} were plated through spread plating techniques on modified Charcoal Cephoperazone Desoxycholate Agar (mCCDA), prepared in house (*Campylobacter* selective agar, LAB M, LAB 112, with Cefoperazone and Amphotericin, LAB M, X212).

2.2.4.2. Enumeration of Campylobacter in caecal content

Using a sterile instrument and aseptic techniques, 1 gram of the caecal content was removed and added to 9ml of MRD. The solution was mixed using the device vortex for 30 seconds and then serial dilutions were prepared using MRD. From each dilution (10^{-1} to 10^{-7}) 100µL was plated using the same plating techniques as for neck skins samples.

2.2.4.3. Bacteria identification and bacteria count

All plates were incubated under microaerobic conditions (MACS-VA 1000- microaerobic workstation: Nitrogen 80%, Hydrogen 3%, Oxygen 5%, C. Dioxide 12%) at 41.5°C for at least 48 hours. After incubation, plates were examined and numbers of presumptive *Campylobacter* colonies were counted in order to calculate the number of bacteria per unit of sample. Final results were recorded as colony forming unit per gram of neck skin or caecal content (cfu/g). The detection limit of this protocol, according to the dilution factor used, was 100 cfu/g.

As reported by other authors, direct plating was considered sufficient for isolation of *Campylobacter* in samples expected to be contaminated with many viable bacteria, in relatively unstressed conditions. Direct plating provided also an estimation of numbers of *Campylobacter* present, quantifying the level of contamination in the samples (Hald, Wedderkopp et al. 2000; Musgrove, Berrang et al. 2001; Kuana, Santos et al. 2008; Rodgers, Clifton-Hadley et al. 2010).

From all samples, presumptive *Campylobacter* colonies were identified directly from mCCDA plates by their typical appearance (grey, flat, irregular and spreading colonies), by microscopic examination after Gram Staining (Gram negative curved rods) and an inability to grow in aerobic conditions (HPA 2011).

2.2.4.4. Campylobacter species differentiation

From each *Campylobacter* positive sample, up to 5 presumptive colonies of different morphologies, were retained for later typing. Each single colony was sub-cultured onto Columbia horse blood agar (CAB) (Columbia Agar Base, LAB M, LAB 001 plus Horse Blood Defribinated, Oxoid, SR00500) and incubated under microaerobic conditions at 41.5°C for a further 48 hours. According to the work schedule, colonies were either put into Microbank vials (Pro-Lab Diagnostics) and stored at -70°C, or immediately processed for identification of species.

From both fresh isolates or ones recovered from frozen beads, chromosomal DNA was extracted by boiling a loop of the cultured colonies in 300µl of Chelex solution (Chelex-100,Bio-Rad) for 10 minutes (Walsh, Metzger et al. 1991). The solution was then centrifuged at 13,000 rpm for 3 minutes (Mikro 20, Hettich, Zentrifugen), to form a pellet containing bacterial cell debris. Fifty µl of the supernatant, containing the suspended DNA, was collected and added to a 450µl of fresh solution containing sterilised distilled water, and the solution was stored at 4°C.

After the DNA extraction, isolates were confirmed and identified to species level *(C. jejuni or C. coli)* using a multiplex PCR as described by Klena et al. based on differences in the *lpxA* gene (Klena, Parker et al. 2004). Isolates negative for amplicons with the *lpx* PCR

were analysed with a PCR assay specific for the genus *Campylobacter* on the basis of 16S rRNA gene sequences (16S rDNA), to confirm that they belonged to the genus *Campylobacter* (Linton, Owen et al. 1996; Katzav, Isohanni et al. 2008). For its close phylogenetic relationship to *Campylobacter* (Lehner, Tasara et al. 2005), its high prevalence reported on broiler carcasses and its potential as an emerging food borne pathogen (Son, Englen et al. 2007), a further PCR for identification of the genus *Arcobacter* (Gonzalez, Garcia et al. 2000) was run on all isolates negative for *Campylobacter.* Primers used and a more detailed description of the typing methods are described in Chapter 5.

2.2.5. Data management and data analysis

Logarithmic transformation was applied to all welfare indicator`s variables: house mortality, rejections, pododermatitis and hock burn and analysis of variance and student t-tests were used to examine differences in welfare indicators among the production systems. Chi-squared χ2 test was used to investigate the association between welfare indicators and *Campylobacter* colonisation. A p value of 0.05 was considered significant. Confidence intervals of all welfare variables were calculated considering the clustering of birds within the farms.

All colony-forming units results were transformed to $\log_{10}$ counts per gram before statistical analysis. Samples in which *Campylobacter* was detected below the enumeration limit were assigned to the value 1 to allow the calculation of the $\log_{10}$ value. The distribution of the enumeration values was highly skewed also after the log transformation and non-parametric statistical analysis was conducted. The chi-squared χ2

test was used to compare the *Campylobacter* prevalence between the production systems, and to investigate the association between batch colonisation and carcasses contamination. Similarly, Kruskal-Wallis rank test and 2-sample Wilcoxon rank-sum (Mann-Whitney) test were used to compare microbial counts. Confidence intervals for *Campylobacter* counts were calculated considering the clustering of birds within the farm. A p value of 0.05 was considered significant.

Except when confidence intervals are provided for an estimate of welfare parameters and *Campylobacter* counts, the analyses carried out in this study have not considered the hierarchical structure of the data (bird>batch>farm) and the clustering of birds within batch/farm/slaughterhouse.

In the analysis, the potential effect of risk factors such as farm and flock parameters, farms practice and other slaughterhouse factors were considered individually. Nevertheless it appeared that thinning, farm and flock size, stocking density, breed, weight, sex, age of birds and killing order are associated with the risk of *Campylobacter* colonisation and carcass contamination, the confounding effect of such factors was not controlled in the analysis. However, the main aim of this study was to identify which production system was posing a bigger risk to the final consumer, and the results presented in this study reflected all the combined effects that were inextricably related to each broiler production system. The statistical analysis was carried out using Stata version 9 (StataCorp 2005).

2.3. RESULTS

From August 2010 till July 2012, a total of 76 batches of broiler chickens (from 46 different farms) were sampled at slaughter. These batches comprised of 26 Standard, 21 RSPCA Freedom Food reared indoor, 11 Improved Welfare and 18 RSPCA Freedom Food reared free range.

2.3.1. Slaughtering operations

Batches from the four broiler production systems were processed in three different high volume slaughtering plants. All the three plants were visited and no major differences were observed in the processing: 155 birds per minute were processed in plant A, 130 birds per minute in plant B and 145 birds per minute in plant C. On arrival at the premises, the birds were unloaded, shackled, stunned (by electrical stunning bath in both Plants A and C or using gas method in Plant B), slaughtered via automatic neck cutter, scalded in order to facilitate the removal of feathers, de-feathered mechanically by rotating rubber fingers' plucking machines, automatically eviscerated (with intestinal pack suspended separately from the back of the carcass), washed and finally cooled for 75 minutes to 3 hours in an air-chiller system.

In all three plants, the final inside/outside washing of carcasses was carried out with cold water with added chlorine dioxide according to the British Standards 12671: 2000. The concentration was 0.20 ppm in plant A and between 0.25 to 0.50 ppm in plants B and C. Data accessible for plant C, showed that 1,550,000 litres of water were used per day at

the final washing stage, with 11.5 litres of water used per carcass (Personal communication from the QA plant department).

The night cleaning procedures were relatively standardised in all the 3 plants visited. An initial removal of the organic matter with high-pressure water was followed by the application of the detergent. A deep rinse followed by a final application of the disinfectant completed the night cleaning operations. No swabs were taken to verify the efficacy of the night cleaning and disinfection procedures against *Campylobacter.* Microbiological checks to verify the effectiveness of the cleaning and disinfection operations to remove *Campylobacter,* were performed by the FBO in plant C on crates and modules used for the transport of the animals. 90% of the swabs taken after the cleaning and disinfection procedures tested positive for *Campylobacter* (Personal communication from the QA plant department). The ineffectiveness of the cleaning and disinfection operations to remove *Campylobacter* contamination from the transport crates was explained (by the QA Department), by the fact that swabbing of crates and modules was carried out immediately after the release of disinfectant, without allowing time for the chemical to act.

During the sampling, slaughter operations and hygiene standards were assessed and recorded. No contamination incidents were observed at any stage during any sampling. The summary of the three slaughterhouses' features is showed in table 2.4.

	Plant A	Plant B	Plant C
Type of birds	ST & FI	IW	FR
Speed line	155 birds/min	130 birds/min	145 birds/min
Stunning method	Electric	Gas	Electric
Temperature of scaldering tanks	52°C	55.6°C	54°C
Time in the Air Chilling (Min)	75	80	180
Carcasses temperature at the exit of the air chilling	<10°C	<10°C	<3.5°C
Chlorine dioxide added to mains water	0.2 ppm	0.2-0.5 ppm	0.2-0.5 ppm

Table 2.4: Features summary of the 3 slaughterhouses investigated

2.3.2. Welfare indicators

2.3.2.1. Welfare indicators by production system

Currently, lesions such as pododermatitis, hock burn, breast dermatitis, house mortality and rejections at slaughter are considered as indicators of housing conditions and of the general welfare of the birds. Also in this study, farm parameters and welfare indicators were recorded to obtain an overall assessment of the animal welfare among the different commercial production systems. Analysis of variance test showed significant differences between the production systems, for all welfare indicators considered (house mortality, rejections, pododermatitis and hock marks) ($p<0.0001$).

Farm parameters and welfare indicators by production system, calculated considering the clustering of the birds within farms, are summarised in Table 2.5. Furthermore, welfare indicators by production system are shown in Figure 2.1 and Figure 2.2.

Farm and welfare Parameters	Standard System	Freedom Food Indoor	Improved Welfare System	Freedom Food Free Range
No. of batches sampled	26	21	11	18
Breed	Ross 308	Hubbard JA57	Ross 308	Hubbard JA57
Average flock size	112728	44678	128629	43542
Number of houses	4.36	2.95	8.64	4.13
Feed mill	A	A	B	C
Stocking density (kg/m²)*	38.490 (37.832 – 39.149)	31.371 (30.125 – 32.518)	30.088 (28.644 – 31.531)	31.067 (29.692 - 32.441)
Age at slaughter (days)*	37.846 (36.881 - 38.812)	48.800 (48.516 – 49.084)	38.454 (36.313 – 40.596)	56.278 (56.078 - 56.478)
Weight at slaughter (kg)*	2.208 (2.153 - 2.262)	2.077 (2.015 - 2.138)	2.292 (2.136 - 2.448)	2.224 (2.157 - 2.292)
House mortality (%)**	2.530 (2.225 - 2.877)	1.355 (1.042 - 1.762)	3.166 (2.359 - 4.248)	1.648 (1.155 - 2.352)
Rejection (%)**	0.503 (0.408 - 0.618)	0.268 (0.205 - 0.351)	1.217 (1.003 - 1.475)	0.327 (0.222 - 0.482)
Pododermatitis (%)**	16.858 (12.715 - 22.353)	1.645 (1.053 - 2.570)	44.050 (31.772 - 61.072)	14.604 (9.137 - 23.342)
Hock burns (%)**	6.099 (4.275 - 8.701)	1.229 (0.956 - 1.579)	8.853 (3.39 - 23.122)	4.548 (2.737 – 7.557)

Table 2.5: Farm parameters and welfare indicators by production system.

Continuous variables are reported as mean or geometrical mean** (95% Conf. Interval with farm as the cluster factor)*

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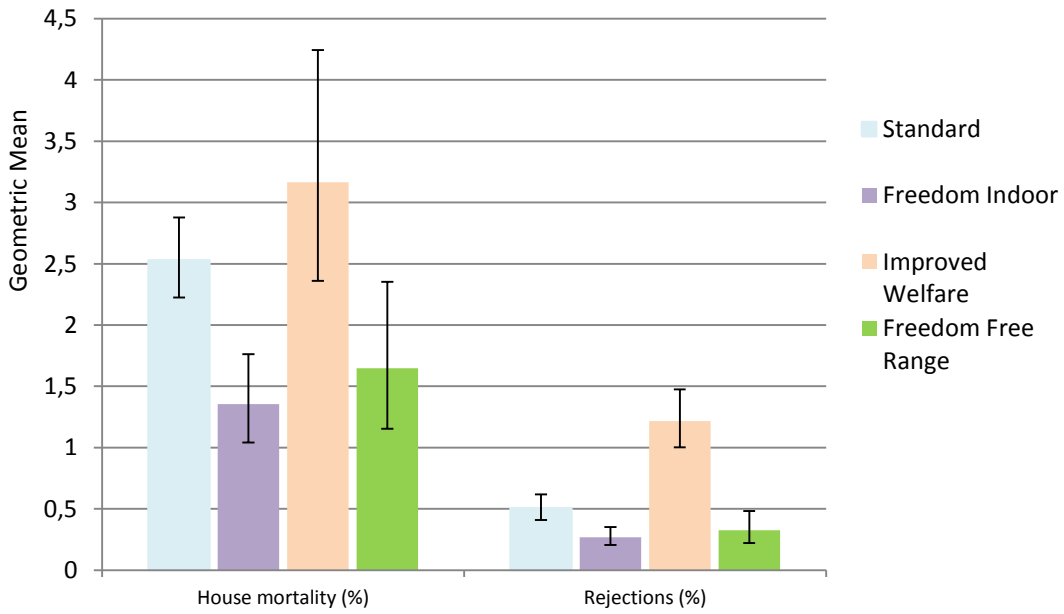

Figure 2.1: Summary of welfare indicators: house mortality (%) and rejections (%) by production system including 95% CIs calculated with farm as the cluster factor

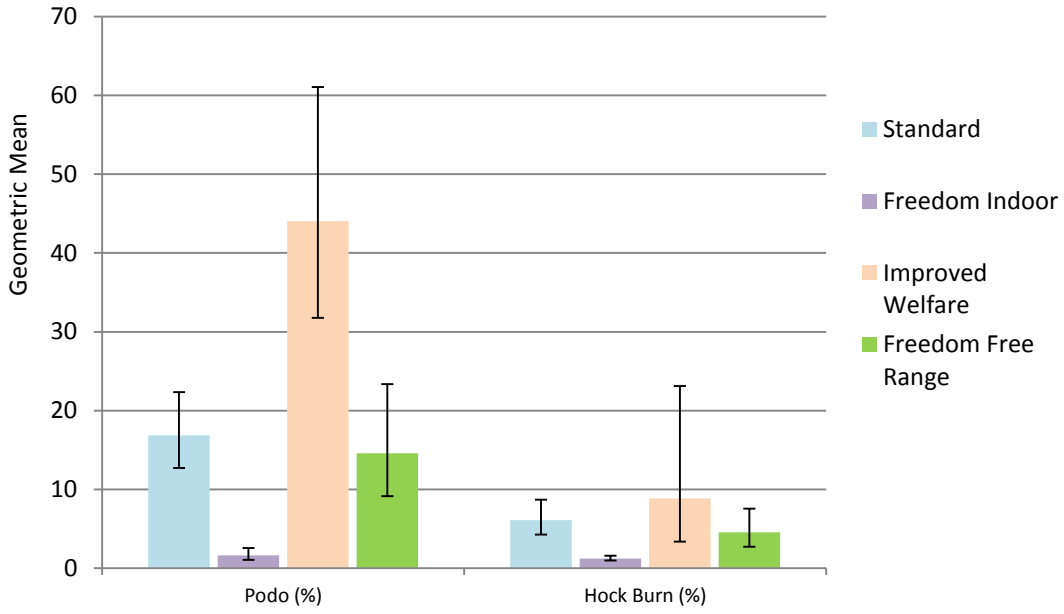

Figure 2.2: Summary of welfare indicators: pododermatitis (%) and hock burn (%) by production system including 95% CIs calculated with farm as the cluster factor

2.3.3. Prevalence and quantification of Campylobacter colonisation

2.3.3.1. Campylobacter prevalence within batch

All the samples were analysed individually. In contrast to other studies, no low prevalence within batch was observed in any production system (Heuer, Pedersen et al. 2001; Allen, Bull et al. 2007; Hansson, Forshell et al. 2007; Jorgensen, Ellis-Iversen et al. 2011). Within the 10 caecal samples collected per batch, all samples either tested all positive or all negative.

2.3.3.2. Campylobacter prevalence throughout the whole broilers population

Within the whole population (760 broilers), a total of 744 individual caecal samples were processed as 16 caecal samples were erroneously missed during sampling. Nineteen caecal samples belonging to two different batches (ST9 and FR8) were excluded from the analysis because of excessive contamination due to lack of antibiotics in the *Campylobacter* growth medium. CFU estimation was made in 725 caecal samples: 440 samples (61%) were positive and 285 (39%) were negative for *Campylobacter*.

2.3.3.3. Campylobacter prevalence within the production systems

Among the 76 batches sampled, a high *Campylobacter* prevalence (61%) was observed within the broiler population studied. The prevalence of *Campylobacter* positive batches was significantly different between the commercial production systems investigated (Chi-squared $\chi 2_{df=3}$ 157.337; $p<0.001$). Higher prevalence (100%) was reported among the Improved Welfare system, with the lowest prevalence (33%) observed in the Freedom system reared indoors (Table 2.6).

	Standard System	Freedom Food Indoor	Improved Welfare	Freedom Food Free Range	Total
Number of broiler batches sampled	26*	21	11	18	76
Number of colonised batches **% Prevalence**	14 56 (50 – 62)	7 33 (27 – 39)	11 100	14 78 (70 – 83)	46 61 (57 – 64)

Table 2.6: Prevalence of Campylobacter colonised broiler batches by production system (95% Conf. Intervals) (Enumeration issue: one batch (ST9) excluded from the analysis because of excessive contamination of plates due to lack of antibiotics in the Campylobacter growth medium)*

2.3.3.4. Campylobacter quantification among production systems

Enumeration results on caecal samples indicated clearly that when batches were colonised by *Campylobacter*, high numbers of microorganisms were observed in the chickens' caecal contents. Although high counts were reported in all production systems, variations were observed. The production system with higher prevalence, the Improved Welfare system had also a higher count. Log cfu/g mean per production system, calculated considering the clustering of birds within the farm, are showed in Table 2.7 and showed in Figure 2.3.

Production Systems	Mean log cfu/g	95% Conf. Interval
Standard	4.300	3.135 - 5.466
Freedom Food Indoor	2.591	-0.441 - 5.623
Improved Welfare	7.971	7.634 - 8.309
Freedom Food Free Range	5.701	4.026 - 7.3769

Table 2.7: Campylobacter mean log cfu/g in caecal content per production system (95% Conf. Intervals calculated with farm as cluster factor)

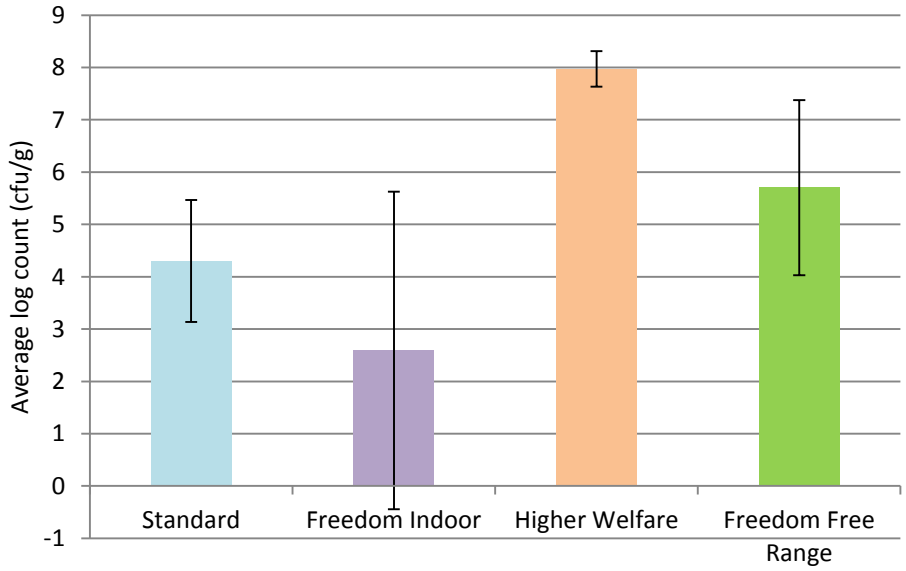

Figure 2.3: Campylobacter quantification in the caecal content in the different broilers' production systems including 95% CIs calculated with farm as cluster effect.

Equality of populations test (Kruskal-Wallis test) showed significant differences between the production systems ($\chi 2_{df=3}$ = 117.507, p<0.0001). When the different systems were compared pairwise statistically significant differences between all groups were identified (p<0.0001), with the exception of Standard versus Free Range (Two-sample Wilcoxon rank-sum test-Mann-Whitney z= -1.746; p=0.081).

2.3.3.5. Campylobacter counts on individual caeca samples

The highest individual count on caecal content was found in a sample collected from a Standard bird (6.50E+09 cfu/g). Also the lowest number of *Campylobacter* in a caecal sample (2.00E+03 cfu/g) was reported among the Standard production system. Campylobacter enumeration results on individual caecal samples by production system are reported in Table 2.8 and shown in Figure 2.4.

Campylobacter Enumeration (cfu/g)	Standard System	Freedom Food Indoor	Improved Welfare	Freedom Food Free Range	Whole Population
<99	105	140	0	40	285
100-999	0	0	0	0	0
1000-9,999	5	0	0	0	5
10,000-99,999	4	0	0	1	5
100,000-999,999	1	2	0	9	12
1,000,000-9,999,999	10	6	10	12	38
10,000,000-99,999,999	55	23	41	77	196
100,000,000-999,999,999	46	35	55	28	164
> 1.00+09	12	3	3	2	20

Table 2.8: Campylobacter counts on individual caeca by production system

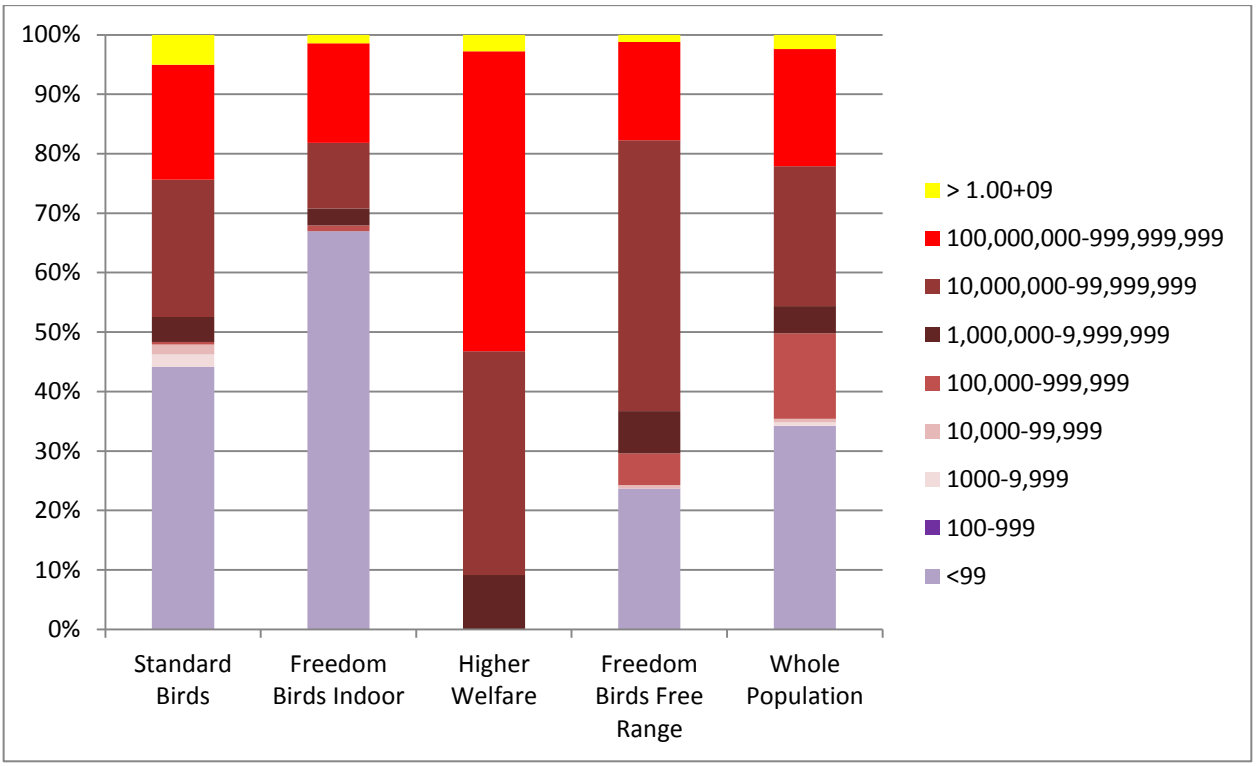

Figure 2.4: Percentage of Campylobacter counts present on caecal samples among the production systems

2.3.4. Association between breed used and flock colonisation

Univariable analysis showed a significantly higher *Campylobacter* prevalence among the faster growing breed (Ross 308) (Chi-squared $\chi 2$ test = 22.853; $p<0.001$). Caecal samples collected from higher growth birds reported also a significant higher count compared to the slow growth birds (Hubbard JA57) (Two-sample Wilcoxon rank-sum, Mann-Whitney test $z = 5.873$; $p<0.0001$).

Figure 2.5 summarised *Campylobacter* caecal counts reported in Ross 308 and Hubbard JA57 birds, considering the clustering of birds within farm.

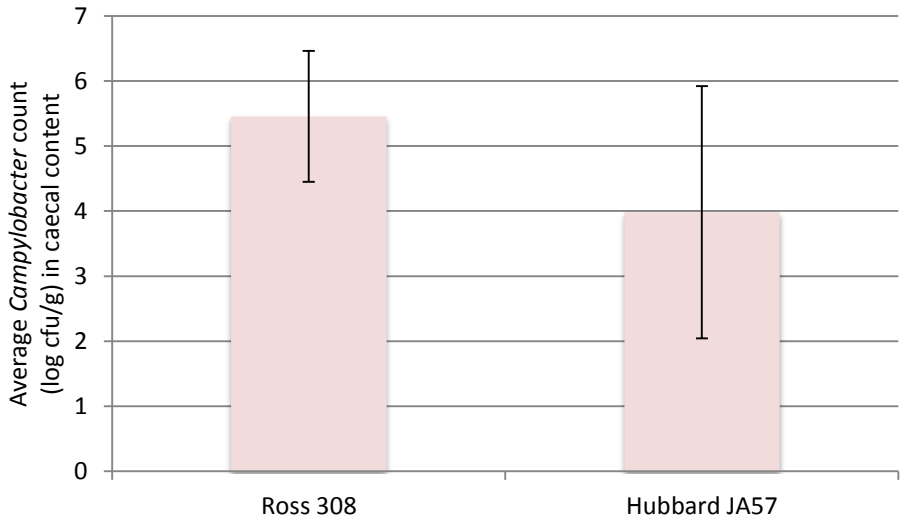

Figure 2.5: Campylobacter quantification in the caecal content according to the breed used including 95% CIs calculated with farm as cluster effect

2.3.5. Association between welfare indicators and batch colonisation

Among the sampled batches, *Campylobacter* infection was associated with an increased prevalence of mortality ($t_{df=665}$ = -7.8607, p<0.0001), rejections ($t_{df=665}$ = -4.7519; p<0.0001), pododermatitis ($t_{df=693}$ = -11.4641, p<0.0001) and hock burns ($t_{df=693}$ = -7.0829p<0.0001). However, when the clustering of the birds within farm was taken in consideration, it was noted that the presence of *Campylobacter* was associated only with the level of pododermatitis (Figure 2.6).

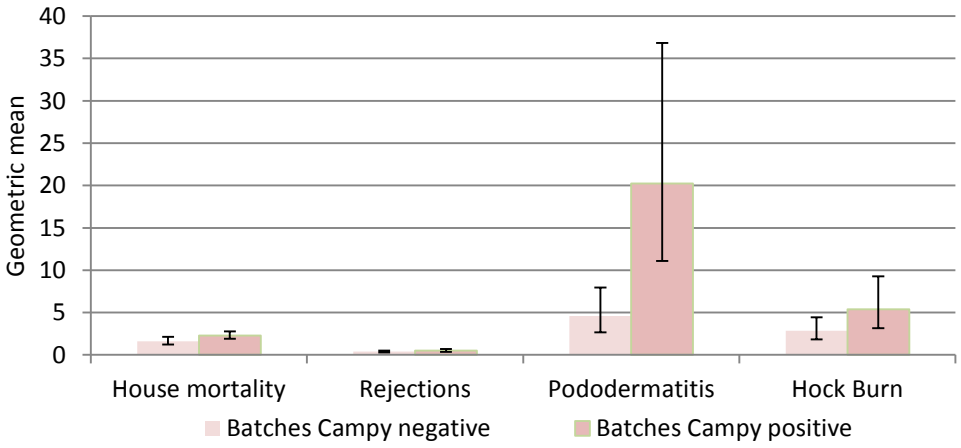

Figure 2.6: Percentage of house mortality, rejections, pododermatitis and hock burn by Campylobacter colonisation status including 95% CIs calculated with farm as cluster effect

When the 2 different breeds were analysed separately, the association between flock colonisation and level of podermatitis was observed only among the faster growing breed (Ross 308) (Figure 2.7).

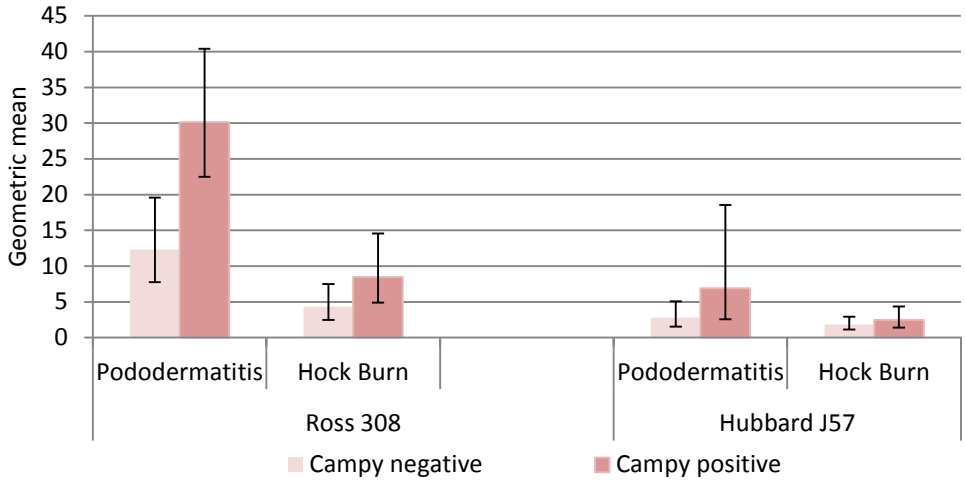

Figure 2.7: Percentage of pododermatitis and hock burns by Campylobacter colonisation status and by breed including 95% CIs calculated with farm as cluster factor

2.3.6. Prevalence and quantification of carcass contamination

Throughout the whole population sampled, 662 neck skin samples were analysed successfully. The prevalence of *Campylobacter* contaminated carcasses reported post air-chilling was 55% (300 negative samples, 362 positive). In contrast to the observation made for the caecal samples where, within the batch, caecal contents either tested all positive or all negative a mix of positive and negative neck skin samples was observed in 16 out of the 76 batches sampled.

2.3.6.1. Association between batch colonisation, carcasses contamination and Campylobacter build-up during production

As reported in other studies, an association between *Campylobacter* batch colonisation and *Campylobacter* carcasses contamination was observed. Within the

sampled carcasses, the prevalence of contaminated carcasses was significantly higher when the batch of origin of the carcass was colonised with *Campylobacte*r (Chi-squared $\chi 2$ test = 395.632; $p<0.001$) (Figure 2.8) (Jeffrey, Tonooka et al. 2001; Herman, Heyndrickx et al. 2003; Hansson, Pudas et al. 2010). Only 6% of broiler carcasses belonging to colonised batches appeared to be *Campylobacter* negative. On the other hand, 92% of carcasses belonging to *Campylobacter*-free batches were *Campylobacter* negative.

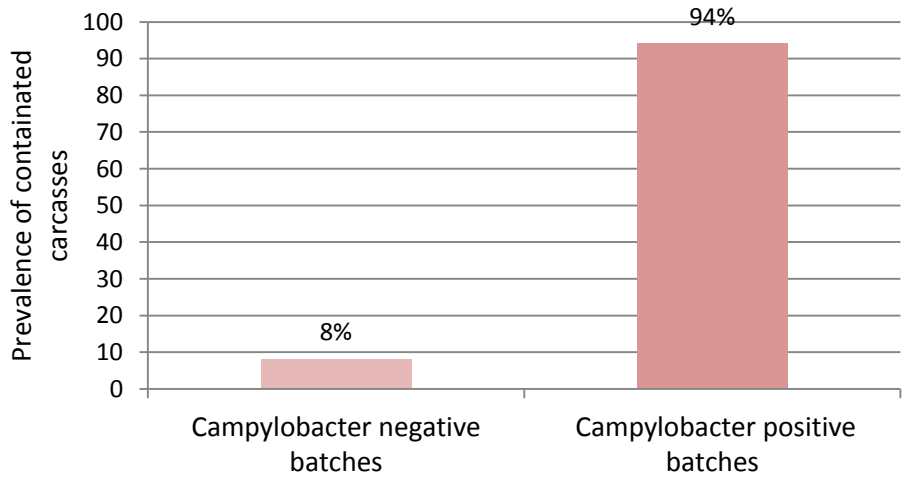

Figure 2.8: Prevalence of contaminated carcasses versus batches' Campylobacter status

Within the 29 *Campylobacter*-free batches, cross-contamination of carcasses occurred in 5 batches (ST2, ST4, ST7, FR11 and ST15). For one negative batch (ST24), neck skin samples were not collected due to an ongoing client audit at the processing plant. Apart from batches FR11 and ST6 that were processed as killing number 3 and 6 respectively, the rest of the cross-contaminated carcasses belonged to batches processed late during the production day (killing order 18, 19 and 14 respectively).

According to the enumeration data, the average number of *Campylobacter* on positive carcasses belonging to slaughter groups with negative caecal samples were significantly lower 0.201 log cfu/g (95% CIs 0.000 - 0.402) when compared to the average count, 2.841 log cfu/g (95% CIs 2.529 – 3.153) reported on positive carcasses belonging to *Campylobacter* positive batches (Two-sample Wilcoxon rank-sum (Mann-Whitney) test z = -19.163; p<0.0001). Figure 2.9 shows *Campylobacter* mean log cfu/g in carcasses according to batch colonisation status including 95% CIs calculated with farm as cluster factor.

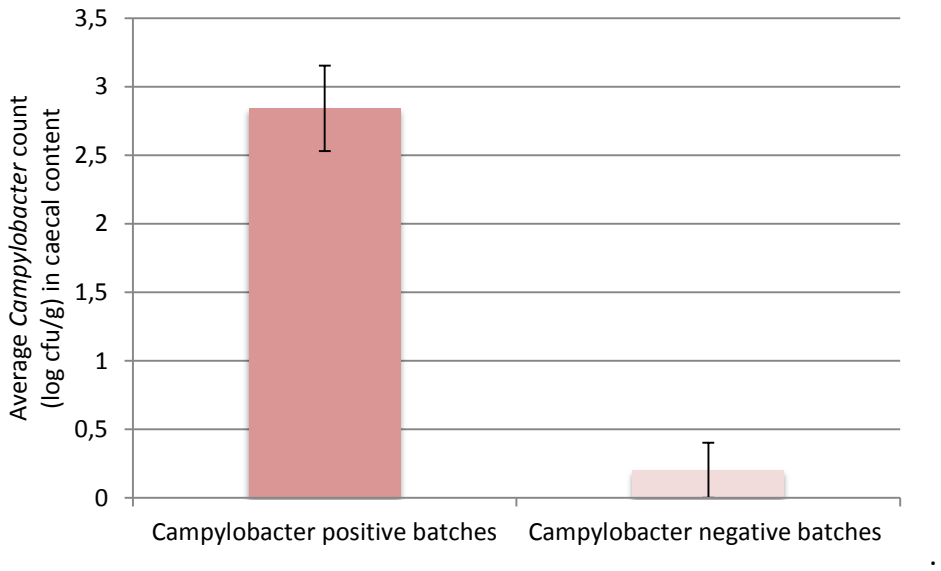

Figure 2.9: Campylobacter quantification in carcass according to batch colonisation status including 95% CIs calculated with farm as cluster effect

The build-up of *Campylobacter* contamination during the production day, was investigated comparing the average of load contamination on carcasses sampled at different times. In order to meet the RSCPA Freedom Food standards, the internal policy of the poultry company was always to process the Freedom Food batches at the start of the production shift. Consequently the effect of the killing order on the load of

carcass contamination was investigated only among the Standard and the Improved Welfare systems. Batches were arranged in 3 groups according to the killing order of processing. In group 1 were included all batches that were slaughtered from killing order 1 to 6, killing order 7 to 14 were included in group 2 and finally all batches processed late during the production day, killing order 14 to 27, were included in group 3. Among the Standard system processed in plant A, a significant difference in the average counts of *Campylobacter* on carcasses was observed among the batches processed early in the morning and late during the day (Two-sample Wilcoxon rank-sum (Mann-Whitney) test $z=-2.790$; $p<0.01$). Among the Improved Welfare birds processed in plant B, a significant difference was observed between the first two groups (Two-sample Wilcoxon rank-sum (Mann-Whitney) test $z=-2.681$; $p<0.01$; $p<0.01$). No batches were included in the third group, as for logistic reason, samples in plant B were collected generally during the morning shift to be posted to the laboratory facilities the same day of collection.

However, when the confidence intervals were provided for an estimate considering the clustering of birds within farms, the order of killing appeared to play a role less marked on the level of carcass contamination (Table 2.9).

Production System	Group (Killing order)	Mean log cfu/g	95% Conf. Interval
Standard system	**1** (1-6) **2** (7-14) **3** (15-27)	1.534 1.905 2.360	0.380 - 2.689 0.974 - 2.836 1.225 - 3.495
Improved Welfare system	**1** (1-6) **2** (7-14)	2.046 3.262	-0.114 - 4.201 2.807 - 3.718

Table 2.9: Carcass Campylobacter mean count log cfu/g versus killing order, showing the build-up of Campylobacter contamination during the production day, including 95% CIs calculated with farm as cluster factor

2.3.6.2. Carcasses contamination within the production systems

A statistically significant association between carcass contamination and production system was observed in this study (Chi-squared $\chi 2_{df=3} = 86.3056$; $p<0.001$). A higher prevalence of contaminated carcasses (86%) was observed within the Improved Welfare production system; lower prevalence of carcasses contamination (31%) was reported among the Freedom Food Indoor system.

Marked variation among the production systems emerged also from the enumeration results. Significant higher *Campylobacter* counts were again reported within the Improved Welfare system (Equality of populations -Kruskal-Wallis test $\chi 2_{df=3} = 76.633$; $p<0.0001$). When the different production systems were compared pairwise (Two-sample Wilcoxon rank-sum test -Mann-Whitney) statistically significant differences were observed between all groups ($p<0.0001$) apart from Standard versus Freedom free range birds (Two-sample Wilcoxon rank-sum (Mann-Whitney) test $z=76.633$; $p=0.821$).

However, when the clustering of birds within the farms was taken in consideration, significant differences between production systems was observed only between the Higher Welfare and the Freedom Food Indoor (Table 2.10).

Production Systems	Mean log cfu/g	95% Conf. Interval
Standard	1.926	1.279 - 2.575
Freedom Food Indoor	0.975	-0.339 – 1.611
Improved Welfare system	2.904	2.211 - 3.597
Freedom Food Free Range	1.963	1.280 - 2.646

Table 2.10: Campylobacter mean log cfu/g in broiler carcasses among the different production systems including 95% CIs calculated with farm as cluster factor

2.3.6.3. Campylobacter counts on individual broiler carcasses

Quantitative data showed clearly that broiler carcasses were contaminated with high numbers of *Campylobacter*. Among the whole population sampled, the percentages of broiler carcasses with enumeration results (cfu/g of neck skin) below 100, between 100-999, between 1,000-9,999 and between 10,000 and 99.999 and above 100.000 were 45.37%, 20.5%, 24%, 8.5% and 0.2%, respectively. In all production systems carcasses with *Campylobacter* counts above 1,000 cfu/g were detected. *Campylobacter* enumeration results on individual broiler carcasses are reported in Table 2.11 and showed in Figure 2.10.

Campylobacter enumeration (cfu/g)	Standard System	Freedom Food Indoor	Improved Welfare	Freedom Food Free Range	Whole Population
<99	93 (41.5%)	141 (68.8%)	12 (13.6%)	54 (37.2%)	300 (45.3%)
100-999	47 (21%)	28 (13.7%)	24 (27.3%)	37 (25.5%)	136 (20.5%)
1,000-9,999	58 (25.9%)	33 (16.1%)	34 (38.6%)	43 (30%)	168 (24.4%)
10,000-99,999	25 (11.2%)	3 (1.5%)	18 (20.5%)	10 (6.9%)	56 (8.5%)
>100,000	1 (0.5%)	0 (0%)	0 (0%)	1 (0.7%)	2 (0.3%)

Table 2.11: Campylobacter counts on individual broiler carcasses by production system

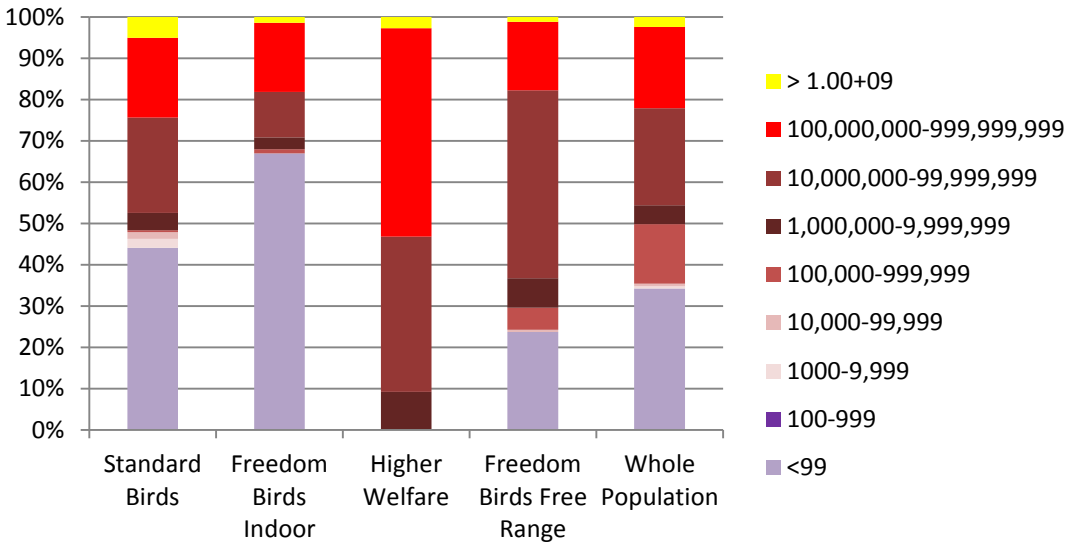

Figure 2.10: Percentage of Campylobacter counts present on neck skin samples among the different production systems

2.4. DISCUSSION

The primary goal of this study was to compare the prevalence of *Campylobacter* in four different commercial broiler production systems in the UK. The fact that all the systems belonged to the same broiler producer may limit the representativeness of the sampling. However, it minimises the effect of the differences in farm and processing management practices. Although the sample size was adjusted for the clustering effect of the farm, except when confidence intervals were provided for an estimate of welfare parameters and *Campylobacter* counts, the analysis presented here does not take into account the clustering effect of the batch or the farm of origin or the premises of slaughter, and significance levels were not always supported by confidence intervals presented considering the clustering of birds within farm.

Overall, 61% of *Campylobacter* colonised batches was reported in our study. However, this high percentage was lower than that reported in the first study carried out by EFSA at European level. The EFSA study, carried out in 2008, found an average prevalence in the EU member states of 71.2%, compared to an average of 75.3% of *Campylobacter* positive batches in the UK broilers population (EFSA 2010a). Taking into consideration the reduced number of batches investigated, the increased prevalence of negative batches reported in our study may be the result of the increased participation of slow growing breeds in the study compared with the national population though one may also hope that this is an encouraging result for the UK poultry producers. It demonstrates clearly that it is achievable to rear *Campylobacter*-free flocks in the current UK poultry industry. Furthermore, knowing that there is scope for

improvement, it should make the scientific community more eager to identify the main contributing factors that enable a farm to produce *Campylobacter* negative flocks.

To incentivise the production of *Campylobacter* negative poultry flocks, some European countries, such as Sweden and Denmark, have introduced a financial reward for flocks that the producers are able to send at slaughter *Campylobacter*-free (WHO 2000). In the same way, these countries are penalising the producers by sending, for example their products from *Campylobacter* positive flocks to the frozen retail market (which is less lucrative than the fresh product market) when flocks are tested (Stern, Clavero et al. 1995; Stern, Hiett et al. 2003). The meat from these negative flocks could be also labelled as '*Campylobacter*-free product'. However, there are diverging opinions between the EU countries on the effectiveness of the proposed labelling, as the concern would be that some consumers could misunderstand this information as 'product safe to eat' and as a result the consumers may neglect to follow the basic hygiene measures during the food preparation (EFSA 2008). Another potential issue with this labelling would be that the producers could be selling these products at higher price hence profiteering from the consumer's lack of knowledge. As the consumer could easily mistake this labelling for another measure of quality which would be wrong since products being *Campylobacter*-free is a matter of food safety and not of food quality.

In all production systems investigated, we observed that when infection entered into a poultry flock all animals were colonised. Several authors reported low prevalence within both conventional and extensively reared flocks at slaughter (Heuer, Pedersen et al. 2001; Hansson, Ederoth et al. 2005; Allen, Bull et al. 2007; Hansson, Forshell et al. 2007; Jorgensen, Ellis-Iversen et al. 2011). However, as documented also by other

authors, in this study in all positive batches 100% of colonised birds were reported (Berndtson, Danielsson-Tham et al. 1996; Shreeve, Toszeghy et al. 2000). This finding confirmed that the bacteria are spread easily within the flock. Inside the broiler house, communal sources of eating and drinking together with the normal coprophagic activity of broilers, facilitate chicken colonisation (Herman, Heyndrickx et al. 2003; Messens, Herman et al. 2009).

Our results suggest significant differences in *Campylobacter* prevalence within the different commercial production systems. Higher prevalence of batch colonisation was observed among the Improved Welfare system. Some batches from Standard and Freedom Food farms (both reared indoors and free range) were found to be negative at slaughter, on the other hand all batches belonging to the Improved Welfare scheme tested positive.

Findings from this study highlighted that birds reared indoors under higher welfare standards but with a fast growing breed, Ross 308 (Improved Welfare system), had a higher prevalence of *Campylobacter* compared to the slower growing breed, Hubbard J57, when grown at the same stocking density and under the same higher welfare standards (Freedom Food system Indoor). Interestingly, when comparing the Improved Welfare system against the Standard system throughout non-parametric analysis, both rearing the same breed of birds (Ross 308) but under different welfare conditions, it was observed that birds reared in the enriched environment and at lower stocking density had a higher *Campylobacter* prevalence. From this finding one could suspect that higher welfare standards were associated with an increase in *Campylobacter* prevalence. However, after discussion with the company veterinarian it emerged that in the Improved Welfare production system, all broiler houses were divided in 2 parts:

one part (which accounted for 10% of the bird population) kept the cockerels that were sent to slaughter at 30-35 days, and in the largest part (which accounted for 90%) were mixed gender birds that were slaughtered at 40-42 days. As almost all batches tested from this production system were subjected to the above thinning process (only one set of samples, HW 10, was collected from the first group) a potential explanation to this high prevalence of batch colonisation in the Improved Welfare system could be the negative impact of the thinning process on *Campylobacter* colonisation (Hald, Rattenborg et al. 2001). In the analysis, the potential effect of the thinning practice and of other potential risk factor was considered only at univariable level. Nevertheless, it is appeared that flock and farm parameters (such as thinning, farm and flock size, stocking density, breed, weight, sex and age of birds) played a role in the risk of *Campylobacter* colonisation, the effect of confounders was not controlled using advanced statistical modelling. In fact, larger flock sizes and a significantly higher number of houses observed among the Improved Welfare farms may have facilitated further the colonisation of batches in this production system. Indeed, in several studies, large flock size and large number of houses have been reported as risk factors for *Campylobacter* colonisation of broilers flocks (Stern, Clavero et al. 1995; Berndtson, Emanuelsonb et al. 1996; Stern, Reiersen et al. 2005; Barrios, Reiersen et al. 2006; McDowell, Menzies et al. 2008). It is clear that more animals require more resources in the farm such as staff, handling, feed, etc. Also large flocks are transported to slaughter in different lots, often spread over few days, therefore increasing the sources of *Campylobacter* colonisation for the remaining birds. Furthermore, it only takes one house to become positive to provide an important reservoir of infection for all the remaining houses.

To point out the effects of each farm and welfare parameters on *Campylobacter* incidence, advanced modelling including the above possible risk factors and random or mixed effects for the role of multilevel clustering is needed. Ultimately only experimental conditions could clarify the effect of the different risk factors but they are not easily implemented at the scale of operations of the contemporary poultry industry. Indeed the prevalence results obtained in our study reflected all the combined effects that were inextricably related to each broiler production system.

It is clear that additional challenges emerge when working with the industry and their daily operations, as agreements are occasionally changed due to unforeseen situations or business needs. In our study, for example, the thinning practice in the Improved Welfare farms was not factored in when the project was designed, and it was only brought to light after data collection. However, the main aim of this study was to identify which production system was posing a bigger risk to the final consumers. After the analysis of the 76 batches, it appeared that the Improved Welfare system with all its characteristics was the system which had the highest *Campylobacter* prevalence, and therefore potentially causing the major threat to human health. Consequently, it is important to identify which practices and which factors among each production system are responsible for the entry and the spreading of *Campylobacter* into the poultry flock.

Supermarkets set their requirements to respond to consumer demand. In the case of the Improved Welfare system the intention was to target their products to shoppers who are more concerned about animal welfare issues. However, the data behind the Improved Welfare scheme showed a high prevalence of house mortality, rejections, pododermatitis, and hock burns. This finding showed that only addressing the stocking

density and the enrichment aspects of the broilers' house, does not bring the results expected to improve the wellbeing of the chickens. Several studies reported that the influence of stocking density on the prevalence of pododermatitis and hock burns is more pronounced in turkeys than in broiler flocks (Kyvsgaard, Jensen et al. 2013). Studies showed that in chickens there are other important factors in the birds' environment such as house size, litter material and condition, litter moisture, climate, diet, temperature, humidity, ventilation, type and management of drinkers, that play an important role in the aetiology of pododermatitis (Martrenchar, Boilletot et al. 2002; Dawkins, Donnelly et al. 2004; Bilgili, Alley et al. 2006; Estevez 2007; Shepherd and Fairchild 2010). Approved schemes adopted by the supermarkets need to take into account all the aspects of poultry management before including a higher welfare labelling of their food products.

It emerged that the presence of *Campylobacter* was associated with the prevalence of pododermatitis and hock burns in both faster and slower growing breeds. However, when confidence intervals were provided for an estimate considering the clustering of birds within the farm, it was noted that *Campylobacter* infection had a stronger association mainly with pododermatitis in the faster growing breed. A relationship between the level of *Campylobacter* colonisation and the levels of hock burns/rejections due to infectious disease in broilers was demonstrated in 2008 by Bull at al. and in a more recent study by Williams at al. (Bull, Thomas et al. 2008; Williams, Sait et al. 2013). In this last study, *Campylobacter* infection was associated with signs of poor gut health resulting in wet litter. It is well known that the wet litter can lead to potential welfare issues such as pododermatitis and hock burns (Harms, Damron et al. 1977; Martland 1985; Kyvsgaard, Jensen et al. 2013). Further research is needed to

investigate the mechanism that link *Campylobacter* with alteration of the physiology of the normal gut ecology, potentially resulting in wet litter. Should the above association between *Campylobacter* colonisation and wet litter be proven, this could have important implications for the poultry industry. As it is recognised that pododermatitis and hock burns are linked to lower flock productivity (Martland 1985), our results indicate that there is a combined benefit for public health and animal welfare in the control of *Campylobacter* infection.

Non-parametric test, used to compare different type of Freedom Food scheme, highlight higher prevalence of flock colonisation and higher bacteria counts in the free-range birds compared to the ones reared indoors. Higher *Campylobacter* prevalence in extensive broiler production systems compared with conventional intensive farming practices was reported in other studies (Heuer, Pedersen et al. 2001; Luangtongkum, Morishita et al. 2006; John Tang Yew Huat 2010). In different studies, greater exposure to environmental challenges and rearing for longer periods were the main factors attributed to this higher prevalence (Heuer, Pedersen et al. 2001; Vandeplas and Dubois-Dauphin 2010; Allen, Ridley et al. 2011). However, Colles et al (2008) in one of their studies rejected the hypothesis that the environment was the major source of infection for free-range chickens on the basis of lack of consistency, based on molecular typing, between strains reported in the free-range broilers and the strains identified in wild birds in the same environment (Colles, Jones et al. 2008). In our study the average age of slaughtering for indoor birds was 49 days, against 55 days for outdoor birds. A higher risk of *Campylobacter* colonisation associated with higher age at slaughter has been reported in several studies, proving that the older the bird is, the longer is the exposure to the potential *Campylobacter* sources (Stern, Clavero et al.

1995; Evans and Sayers 2000; Northcutt, Berrang et al. 2003; Stern, Reiersen et al. 2005; Barrios, Reiersen et al. 2006; McDowell, Menzies et al. 2008; Hansson, Pudas et al. 2010; EFSA 2010b; Colles, McCarthy et al. 2011). Consequently, lowering the slaughtering age could be a measure to reduce *Campylobacter* prevalence in the broiler flocks (Berndtson, Emanuelsonb et al. 1996). However, slaughtering at a younger age might be not viable for financial reasons in particular when slow-growing breeds are used, as birds need to grow to a certain weight to be commercialised. Moreover, the fact that some schemes specify a minimum age for slaughtering for animal welfare reasons (such as 49 days in the Freedom Food Scheme) (RSPCA 2011), eliminates the opportunity to use this control measure in the more extensive systems.

We observed that *Campylobacter* colonisation of the intestinal tract reflected the presence of *Campylobacter* on carcasses. Slaughter batches with caecal negative samples, had significantly more negative neck skin samples compared with colonised slaughter batches (Jeffrey, Tonooka et al. 2001; Lindblad, Hansson et al. 2006; Allen, Bull et al. 2007; Reich, Atanassova et al. 2008; Hansson, Engvall et al. 2010). A positive association between carcass contamination and flock colonisation emerged also from the enumeration data. The average count of *Campylobacter* on broiler carcasses was greater (2.841 cfu/g) in slaughter groups with a high degree of intestinal colonisation. On the other hand, a lower average *Campylobacter* count (0.201 cfu/g) was reported in all cross-contaminated batches (Allen, Bull et al. 2007; Johannessen, Johnsen et al. 2007; Reich, Atanassova et al. 2008; Hue, Le Bouquin et al. 2010). Among the four systems investigated, the Improved Welfare system, with the highest *Campylobacter* prevalence and the highest *Campylobacter* count in caecal content, had also a higher *Campylobacter* prevalence and average counts on carcasses. The above findings are

consistent with the results from other previous studies that identified intestinal colonisation as the main source of carcasses contamination (Allen, Bull et al. 2007).

In addition, the high rate of caecal carriage at the time of slaughter is a key factor in the occurrence of *Campylobacter* in the processing environment (Figueroa, Troncoso et al. 2009). Considering that during a production day, in an average UK large slaughterhouse more than 25 different batches can reach the processing plant, it is easy to understand how the build-up of bacteria occurs when *Campylobacter* positive flocks reach the processing plants. In this study, the effects of the accumulation of bacteria during the production surfaced when investigating the bacterial counts on carcasses processed at different times of the day. Nevertheless other confounding factors such as different processing parameters could have played a role on the level of carcass contamination, and bearing in mind that the non-parametric analysis did not take in consideration the clustering of carcasses within the farms/ slaughterhouses, it was found that higher numbers of bacteria on carcasses were reported in batches processed late during the day, compared to the ones processed earlier (EFSA 2010b). It is important to investigate the hygiene procedures such as automated cleaning of machinery, personal hygiene, use of hand wash facilities, use of correct protective clothing and break cleaning that can minimise the build-up of bacteria during processing.

In our research, cross-contamination of carcasses belonging to *Campylobacter*-free batches was observed in plants A and C. In the third plant, plant B, all the batches were *Campylobacter* positive and as a result cross-contamination could not be proven. Several studies showed that it is difficult to avoid the spread of bacteria during processing, and it cannot be only attributed to bad production practices, as it is an

intrinsic factor of the automated slaughtering process. However, in this study cross-contamination was detected in 5 out of 28 *Campylobacter*-negative batches, affecting the 8% of carcasses belonging to negative batches. This figure indicates that high hygiene practices during the operations can mitigate the risk of cross-contamination of the final product for *Campylobacter*-free batches (EFSA 2010b). During the slaughterhouse visits, no hygiene incidents or unacceptable hygiene practices were observed or reported. However, practices such as the use of hose pipes to clean the floor in the plucking or evisceration areas during the production need to be avoided to minimise the creation of aerosols that can facilitate the transfer of bacteria onto the final products. This type of recommendation may seem insignificant when looking at the big picture of the *Campylobacter* world. Nevertheless, such basic hygiene rules would be easy to implement in order to minimise the spread of bacteria in the processing line environment (Viator, Cates et al. 2008). Consequently, it is important that the Regulatory Bodies share across the poultry industry any measure that has any potential impact in the reduction of human infections.

Due to their welfare scheme requirements, Freedom Food flocks (both reared indoors and free range) were always killed at the beginning of the day. As a result, their carcasses were less exposed to the risk of cross-contamination. Among the Freedom birds reared indoors, no evidence of cross-contamination of carcasses belonging to *Campylobacter*-free batches was observed. On the other hand, indications of cross-contaminations were seen among Standard birds processed late during the production day. As data from this study reported a lower *Campylobacter* prevalence between the Freedom Food batches reared indoors, the Freedom Food scheme policy operating in the processing plant and leading to the slaughter of these flocks at the beginning of

the working day, was unknowingly helping to prevent the accumulation of this pathogen in the plant. To know the *Campylobacter* status of the incoming birds and to schedule the processing of batches according to it, can reduce the build-up of the bacteria in the slaughterhouse environment and lower the risk of cross-contamination which can help to preserve the negative status of *Campylobacter*-free flocks.

The proportion of carcasses considered negative by the enumeration method, meaning below the threshold of 100 cfu/g, varied from 13.6% in the Improved Welfare system to 68.8% in the Freedom Food system reared indoors; with an average of 45% of *Campylobacter* negative carcasses reported post air-chilling among the whole population tested. This data revealed that the prevalence of carcasses contaminated with *Campylobacter* at the end of the processing line was very high (55%). This is alarming, as potentially an average of 5.5 carcasses out of 10 broiler carcasses commercialised in the UK market were contaminated with *Campylobacter*. Such high prevalence confirms that broiler meat is a significant vehicle for exposure of the UK consumers to *Campylobacter*. Furthermore the proportion of samples with high counts, above 1,000 cfu/g, varied from 17.6% in Freedom birds to almost 60% among carcasses belonging to the Improved Welfare system, indicating that these broiler carcasses on the supermarket shelves can be contaminated with high numbers of *Campylobacter*. Such carcasses represent a high health risk for consumers, as human campylobacteriosis appears to be particularly associated with exposure to high numbers of bacteria (Lindqvist and Lindblad 2008; Nauta, Hill et al. 2009).

A decade of investments, implementation of new policies, heightened scientific research supported by the UK Government did not deliver the results expected to achieve a sustained reduction in human campylobacteriosis in the country. Therefore

in 2010, in an attempt to break with the year on year increasing trend of human infections, the UK Government published its food policy strategy 'FSA Strategy 2010-2015', with the overall objective being that "Food produced or sold in the UK is safe to eat" (FSA 2010). The main priority of this strategy was to tackle, over a 5-year period, *Campylobacter* in chickens, setting targets to reduce the number of the most highly contaminated broiler carcasses at the end of the slaughtering process. Targets, based on *Campylobacter* counts, are being monitored using a banding approach, where broiler carcasses collected post air-chilling are grouped into 3 bands according to whether the *Campylobacter* counts are above or below the three following values: <100 cfu/g, 100-1,000 cfu/g and >1,000 cfu/g. Progresses are being monitored against the baseline data, collected from the survey 'on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses' carried out in the EU in 2008. This survey had as main objective to obtain through harmonised sampling schemes, set in the Commission Decision 2007/516/EC, comparable data among the EU states (FSA 2010; EFSA 2010a). The UK Government's goal was to reduce the percentage of the most highly contaminated carcasses, with more than 1,000 cfu/gram, from a baseline of 27% to 10% by 2015. Achievements of the targets were firstly expected with the implementation at farm production level of new bio-security standards through the Red Tractor Assurance Scheme which were put in place from April 2011 among the UK broiler population. Secondly corrective measures are being focused at slaughterhouse level with the use of the 'slaughterhouse self-assessment tool' which measures the effectiveness of the existing systems on *Campylobacter* control and identifies areas through the processing line where remedial actions can be implemented. Progresses towards the 2015 objectives were reviewed in

2013, and the interim data published in September 2013 showed that despite the new implemented bio-security measures, no improvements were yet made in reducing the levels of *Campylobacter* (FSA 2013).

When comparing the enumeration results on neck skins from our investigated production systems against the FSA targets, it emerged that the reported 32.9% of highly contaminated carcasses was way behind the FSA's objective. In particular among the 4 commercial production systems studied, the major issues were seen in the Improved Welfare system. It is well known that until the highly contaminated carcasses are removed from the markets, the infections in humans cannot be lowered (Nauta, Hill et al. 2009; Swart, Mangen et al. 2013).

So far, the above findings show that half way through the FSA strategy, the UK chicken meat leaving the processing plant is currently far from being safe to eat. With the current EU regulation, not allowing measures such as chlorination and other anti-microbial agents or irradiation, there are no corrective actions through the food production chain that can eliminate completely the presence of *Campylobacter* in broiler meat (Dincer and Baysal 2004). Consequently it appears crucial to prevent flock colonisation during the rearing period, to ensure negative flocks are entering into the processing plant, since the high bacteria numbers on final product appear to be strongly associated with the colonisation of the slaughter batch.

Bearing in mind the 'big issues' that Governments, poultry producers, food manufacturers and scientific community are being confronted with when tackling this pathogen, it is crucial that good working partnerships are established to exchange and share resources, findings, and experiences. It is important also to engage with the final

consumer, by increasing his awareness on food safety issues and food hygiene behaviours.

CHAPTER 3

CROSS-CONTAMINATION DURING THE SLAUGHTERING PROCESS

3.1. INTRODUCTION

In the UK, the majority of the poultry abattoirs, in order to cope with an ever increasing meat demand, have moved from manual processing operations to more sophisticated automated machines that speed up the production process. Nowadays a large slaughterhouse can process up to 150,000 birds in a 16-hour shift, with a speed line up to 155 birds per minute. Bearing in mind the proportion of *Campylobacter* colonised flocks that arrive at slaughter and the sheer number of birds that are processed on a daily basis, it is quite easy to see how difficult it is to control the spread of this pathogen during processing.

On arrival at the abattoir, birds are unloaded, shackled, stunned, killed, scalded, de-feathered, eviscerated, washed and cooled. Excluding the cooling stage, the entire slaughtering process from shackling to the entry into the air-chiller can take less than 10-15 minutes. The cooling process in a standard UK poultry slaughterhouse is normally carried out in an air-chiller, where carcasses reach the required temperature within 1 to 3 hours. At the exit of the air-chiller, poultry carcasses normally undergo an additional period of refrigeration in order to reach a temperature allowing them to be maintained within the statutory requirements during further processing ($4^{o}C$)

Different studies have shown that cross-contamination occurs during processing (Allen, Bull et al. 2007; Hayama, Yamamoto et al. 2011). Berrang in 2001 reported that carcasses sampled at the end of the processing line had been contaminated with *Campylobacter* even when the bacteria were not isolated from the chickens on arrival at the abattoir (Berrang, Buhr et al. 2001; Newell, Shreeve et al. 2001; Johnsen, Kruse

et al. 2007). Furthermore, it was shown that the subtypes of *Campylobacter* found on the carcasses from colonised birds were not always those which were most prevalent in the gut of the birds (Newell, Shreeve et al. 2001). It is therefore important to ascertain which types of cross-contaminations are occurring during the processing.

Several longitudinal studies have been carried out to estimate which stages during the chicken meat production lead to an increase or decrease in the *Campylobacter* numbers on carcasses (Rosenquist, Sommer et al. 2006). Studies reviewed by Guerin, showed a general decline in the number of *Campylobacter* towards the end of the processing line. More specifically, processes such as scalding, washing and chilling led to a reduction in *Campylobacter* counts on carcasses (Guerin, Sir et al. 2010). Chilling, in effect, could be considered a significant control point for the reduction of carcass contamination. However, the chilling has a limited effect on the number of pathogens on final products if broilers enter into the slaughter process with high levels of *Campylobacter* contamination (Figueroa, Troncoso et al. 2009).

The aim of this study was to investigate the impact of different slaughter operations on carcass contamination in a UK processing plant, to identify how the number of *Campylobacter* changes as carcasses progress through the production line. Furthermore, to better focus potential corrective actions and targets for interventions, it is needed to determine which steps during slaughtering are increasing or decreasing the number of bacteria on carcasses.

3.2. MATERIALS AND METHODS

3.2.1. Study design and sample collection

To understand how the number of *Campylobacter* changes as the carcass progresses through processing, three sampling points were identified along the slaughtering line: after evisceration (EV), after final washing (PW) and post air-chilling (PC). All sampling was carried out in plant A previously described in Chapter 2, with the features summarised in Table 3.1. Plant A was selected for its proximity to the laboratory. The days of sampling were planned ad hoc according to the weekly workload in the laboratory.

Slaughterhouse factors	Plant A
Type of birds processed	Standard and Freedom Food Indoor birds
Speed line	155 birds/min
Stunning method	Electric
Temperature scaldering tanks	52°C
Time in the Air Chilling (Min)	75
Carcass temperature at the exit of the air chilling	<10°C
Chlorine dioxide added to mains water	0.2ppm

Table 3.1: Features summary of the slaughterhouse investigated

On the day of sampling, one neck skin sample from each sampling point was collected from every batch processed during the morning production shift. Caecal samples were also collected at evisceration (one caecal sample from each batch) to estimate the bacterial load of incoming batches. Only intact caeca were collected and neck skin samples were obtained from carcasses free from any visible faecal contamination. After collection, samples were kept individually into a sterile bag, in refrigerated conditions until processing. Samples were processed within 24 hours from collection.

Figure 3.1: Schematic flow chart of the slaughtering process

3.2.2. Campylobacter detection and enumeration in caecal and neck skin samples

For caecal and neck skin samples, the methods for *Campylobacter* enumeration were based on the ISO method 10272-2:2006 (ISO 2006). Enumeration methods for these samples have previously been described in Chapter 2.

3.2.3. Data management and data analysis

The unit of the analysis was the 'slaughter batch', defined as a group of chickens from the same farm delivered to the slaughterhouse at the same time in the same vehicle. All colony-forming unit (CFU) results were transformed to $\log_{10}$ counts per gram before statistical analysis. Samples in which *Campylobacter* was detected below the enumeration limit were assigned to the value 1 to allow the calculation of the $\log_{10}$ value. The distribution of the enumeration values was highly skewed also after the log transformation, and non-parametric statistical analysis was conducted. Chi-squared $\chi 2$ test was used to compare the proportion of positive caecal and neck skin samples in the different sampling points. Similarly, Kruskal-Wallis rank test and 2-sample Wilcoxon rank-sum (Mann-Whitney) test were used to compare microbial counts in the different sampling points. Confidence intervals for *Campylobacter* counts were calculated using bootstrapping with 1000 replications. The *Campylobacter* count in caecal contents was compared to the contamination of carcasses using the correlation. A p value of 0.05 was considered significant. The statistical analysis was carried out using Stata software version 9 (StataCorp 2005)

3.3. RESULTS

Between October 2010 and May 2011 seven 'One Day Sampling' trials were completed. Days of sampling were chosen ad hoc, and samples were collected during the morning shift (06:00-12:30) from the first 9-12 consecutive batches processed over that time. In each sampling day, *Campylobacter* carcass contamination was estimated during the slaughtering process in the 3 sampling points identified.

3.3.1. Prevalence of carcass contamination during the processing

During the seven sampling days, a total of 265 samples were examined. The neck skin contamination rate was 56% (Table 3.2). The *Campylobacter* status of incoming batches, estimated through the examination of 72 caecal samples, showed a prevalence of 39%.

	Post evisceration	Post final wash	Post Chilling	Total neck skin samples
N° of sample positive/N° examined (%)	37/72 (51)	48/72 (67)	23/49 (47)	108/193 (56)

Table 3.2: Prevalence of Campylobacter on broiler carcasses evaluated at the 3 sampling points

The highest rate of *Campylobacter* positive neck skin samples was reported at post wash (67%) (Table 3.2 and Figure 3.2). A statistically significant association between carcasses' positivity and sampling points was not found (Chi-squared $\chi 2_{df=3} = 5.578$;

p=0.061). However, when the sampling sites were compared pairwise, a significant decrease in the number of positive carcasses was observed between samples collected post final washing and post chilling (Chi-squared $\chi 2 = 4.680$; $p<0.05$). Furthermore, when the number of positive carcasses was investigated according to the *Campylobacter* status of the flock (positive or negative), the same trend was noted: the higher number of positive samples was reported post washing (Figure 3.2). Between the negative batches, a significant increase in the number of positive samples moving from EV to PW was observed (Chi-squared $\chi 2 = 4.632$; $p<0.05$).

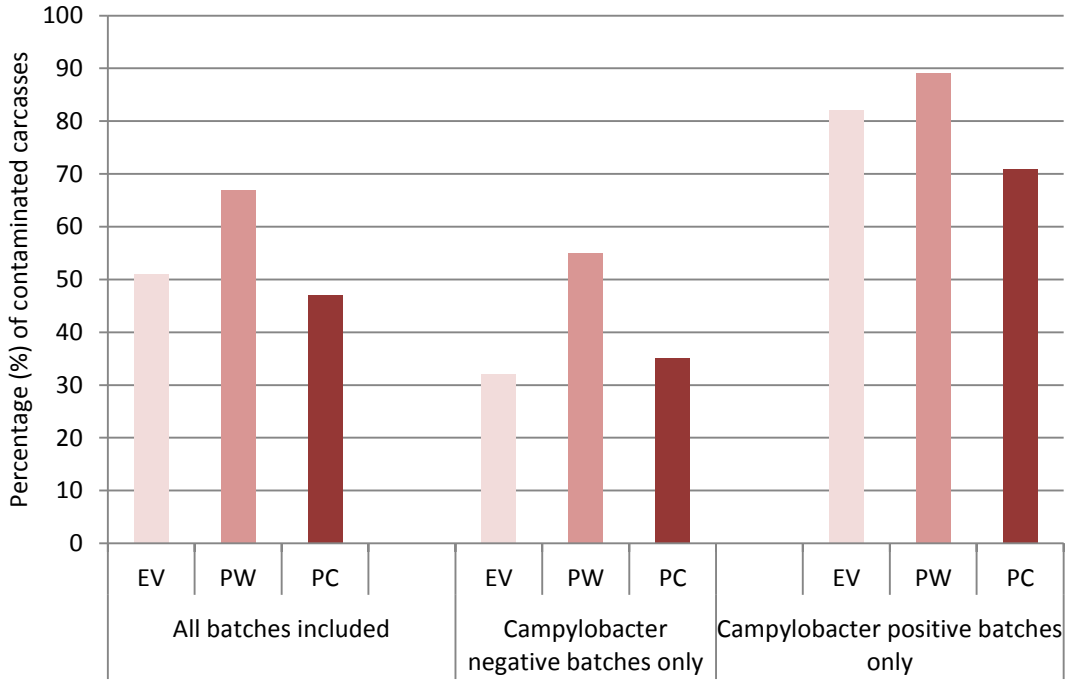

Figure 3.2: Prevalence of contaminated carcasses at different sampling points (by Campylobacter status)

In all three sampling sites, the number of positive carcasses was significantly associated to the batch *Campylobacter* status (EV: Chi-squared $\chi 2 = 17.347$; $p<0.001$; PW: Chi-squared $\chi 2 = 9.011$; $p<0.01$; PC: Chi-squared $\chi 2 = 5.421$; $p<0.05$).

Sampling Day	Batches analysed per day	Number of farms processed per day	Farm ID	Farm type	Age (days)	Podo (%)	Hock burn (%)	Batches processed per farm	Positive batches per farm	Enumeration log cfu/g (average per farm)				Enumeration log cfu/g (average per day)			
										Neck Skin Post EV	Neck skin Post Wash	Neck Skin Post Chill	Caeca	Neck Skin Post EV	Neck skin Post Wash	Neck Skin Post Chill	Caeca
1	12	3	A	ST	40	23.3	37	6	1	1.05	0.83	*	0.74	1.93	1.64	*	3.98
			B	ST	40	30	20	2	2	3.05	3.56	*	7.98				
			C	ST	40	13	27	4	4	2.69	1.89	*	6.84				
2	11	2	D	ST	40	65	27	6	6	2.6	2.89	2.09	6.7	1.78	2.63	1.66	3.93
			E	ST	40	37	21	5	1	0.81	2.31	1.12	0.6				
3	10	2	F	ST	34	25	26	7	0	1.25	1.72	1.09	0	2.08	2.37	1.81	2.61
			G	ST	34	15	20	3	3	3.34	3.35	2.89	6.52				
4	10	1	H	ST	36	22	11	10	0	0	0	0	0	0	0	0	0
5	10	2	I	ST	35	23	35	6	0	0.72	2.59	2.07	0	1.36	2.48	2.23	0.82
			J	ST	39	36	35	4	1	2.33	2.31	2.43	2.04				
6	10	3	K	FI	49	0	0	4	0	1.37	0.62	0.67	0	2.53	1.65	1.7	2.11
			L	ST	41	40	13	3	3	4.51	3.74	3.52	7.05				
			M	ST	35	18	18	3	0	2.1	0.93	1.04	0				
7	9	3	N	FI	49	0	0	2	0	0	3.65	0	0	1.32	2.43	0.81	4.15
			O	ST	38	31	18	4	4	0.5	1.08	0	4.57				
			P	ST	37	36	2	3	3	3.28	3.43	3.27	7.25				

Table 3.3: Summary results (Note: data for day 1, concerning neck skin samples collected post air-chilling, are not available due to a mislabelling issue)*

3.3.2. Enumeration of Campylobacter contamination on neck skin samples at different sampling points

The highest *Campylobacter* contamination rate was reported in samples collected post final wash, and a decrease in the number of pathogens towards the end of the processing line was observed. However, no significant differences were identified between the *Campylobacter* counts from the different sampling points (Kruskal-Wallis test $\chi 2_{df=2} = 2.670$; p=0.263). The same result was also found when the test was carried out individually for each sampling day. The following table shows the *Campylobacter* log mean/g on neck skin by sampling point (Table 3.4).

Sampling point	Mean (log cfu/g)	95% Conf. Interval
Post Evisceration	1.589	1.197 - 1.991
Post Final Washing	1.881	1.544 - 2.217
Post Air Chilling	1.346	0.932 - 1.759

Table 3.4: Mean log colony forming units per grams on neck skin samples at various stages throughout the processing

The mean log colony forming unit per gram in caeca samples was 2.55 cfu/g (95% CI 1.76 - 3.35). In all sampling sites the average log mean per gram was significantly higher when the batch was colonised at caeca level (EV: Two-sample Wilcoxon rank-sum-Mann-Whitney test = -4.827, p<0.0001; PW: Two-sample Wilcoxon rank-sum-Mann-Whitney test = -4.213, p<0.0001; PC: Two-sample Wilcoxon rank-sum-Mann-Whitney test = -3.246, p<0.005) (Table 3.5).

Sampling point	Flock status	Mean (log cfu/g)	95% Conf. Interval
Evisceration	*Campylobacter* negative *Campylobacter* positive	0.839 2.767	0.461 - 1.218 2.208 - 3.326
Post final washing	*Campylobacter* negative *Campylobacter* positive	1.334 2.842	0.952 - 1.715 2.359 - 3.324
Post chilling	*Campylobacter* negative *Campylobacter* positive	0.877 2.279	0.448 - 1.306 1.535 - 3.024

Table 3.5: Mean log colony forming units per gram on neck skin samples at various points throughout the processing according to batch colonisation status

Positive correlations between the number of bacteria in caeca and carcasses were observed in all sampling points. The correlation coefficients (r) were: 0.640 for neck skin samples collected at evisceration ($p<0.0001$) (Figure 3.3), 0.537 for neck skin samples after final wash ($p<0.0001$) (Figure 3.4), and 0.476 for neck skin samples collected post chilling ($p<0.005$) (Figure 3.5).

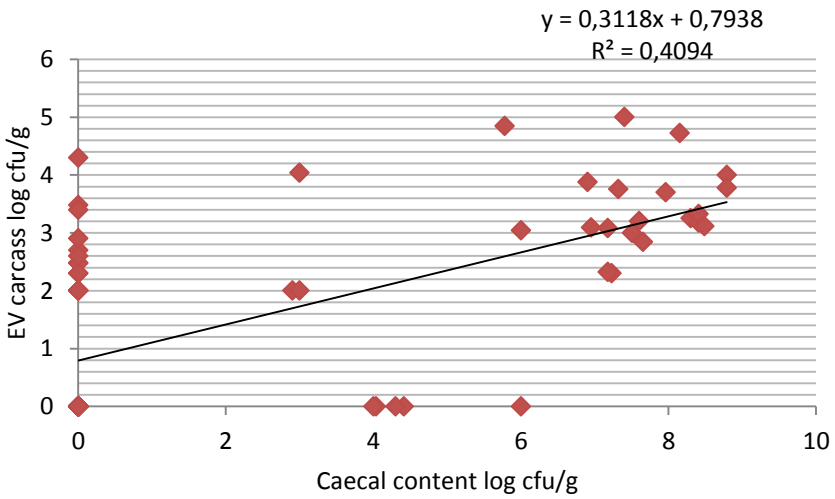

Figure 3.3: Correlation of the number of Campylobacter in caecal content with the contamination of the broiler carcasses at evisceration (EV)

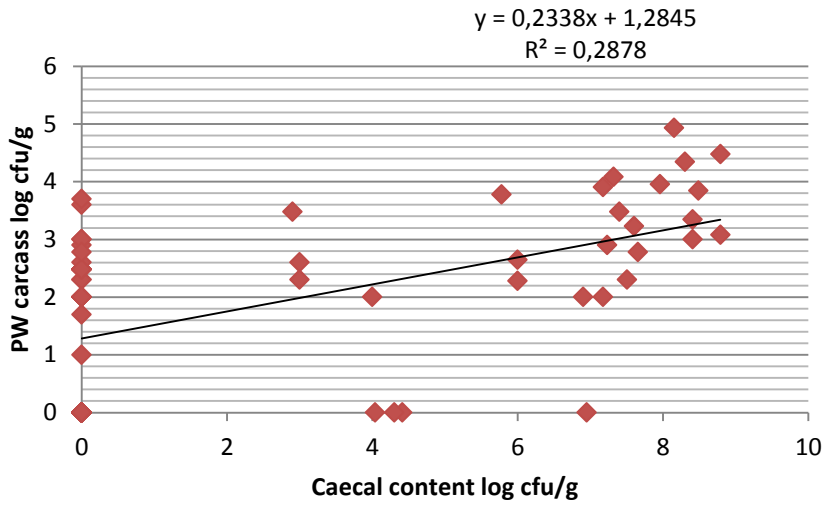

Figure 3.4: Correlation of the number of Campylobacter in caecal content with the contamination of the broiler carcasses after final washing (PW)

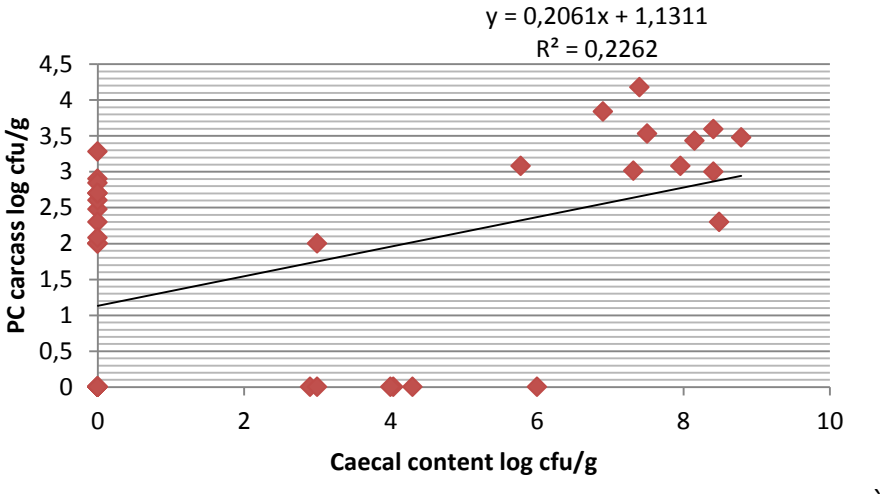

Figure 3.5: Correlation of the number of Campylobacter in caecal content with the contamination of the broiler carcasses post air-chilling (PC)

On the 28th January 2011 (sampling day 4) only one farm was processed during the whole shift. Batches from all five broiler houses reached the slaughterhouse in different lots, and the whole farm was emptied by the end of the morning shift. No

Campylobacter was detected in caeca and neck skin samples. The whole farm (94050 birds) resulted to be *Campylobacter*-free.

In six out of seven sampling days, the first batch of the day was *Campylobacter*-free at caeca level, but still *Campylobacter* was detected in the neck skin samples collected for those first batches. More specifically, 3 out of 6 neck skin samples collected at evisceration from *Campylobacter* negative batches, 5 out of 6 collected at post washing, and 1 out of 5 collected post air chilling were *Campylobacter* positive.

3.4. DISCUSSION

Widespread *Campylobacter* contamination on broiler carcasses throughout the various stages of processing was observed in this study. The overall prevalence of contamination on carcasses, as measured by the contamination of the neck skin, ranged between 47% and 67%, depending on the step investigated. It appears to be a trend with a higher prevalence of carcass contamination and a higher level of contamination observed post final washing. By contrast, after the chilling, there is a trend indicating a decrease in the number of contaminated carcasses and in the *Campylobacter* counts.

Our data showed that carcass contamination was high even at the later stages of processing. Considering the *Campylobacter* prevalence of the incoming flocks (39%), this high prevalence of contaminated carcasses was expected (Hue, Allain et al. 2011). As reported in other studies, a positive association between the average number of *Campylobacter* in caeca and on carcasses was reported in all sampling sites (Herman, Heyndrickx et al. 2003; Allen, Bull et al. 2007; Johannessen, Johnsen et al. 2007; Reich, Atanassova et al. 2008; Hue, Le Bouquin et al. 2010). The levels of *Campylobacter* on broiler carcasses were greater in the slaughter group with intestinal colonisation. On the other hand, a lower average *Campylobacter* count was reported in all cross-contaminated batches. This finding was in accord with previous studies that identified the main source of the *Campylobacter* contamination during the slaughter operations the colonisation of the live flocks (Allen, Bull et al. 2007).

Evisceration has been considered a critical step in carcass contamination (Rosenquist, Sommer et al. 2006), and although in recent years slaughtering techniques and hygiene practices during the operations have improved, the contamination of carcasses from their intestinal contents is not likely to be completely prevented. At the arrival at the processing plant, all batches were weighed and the processing machines were adapted to the average weight of the birds. However natural variation in bird size or improper adjustment of machines could result in viscera rupture, leading to contamination of carcasses (Boysen and Rosenquist 2009), machines (Izat, Gardner et al. 1988; Berndtson, Danielsson-Tham et al. 1996), working surfaces and staff's hands (Jozwiak, Reichart et al.), thereby increasing also the risk for cross-contamination of *Campylobacter*-free carcasses (FAO and WHO 2002). It is important during the processing to take all the necessary measures to minimise the breakage of the chicken intestinal pack. In the past, poultry breeding companies have dedicated a lot of effort to the selection of breeds able to produce uniform flocks in different environments (Aviagen 2012). The uniformity of the incoming flocks, together with adequate slaughterhouse management able to maximise machinery efficiency is a significant step towards achieving less viscera ruptures during the processing.

Although during the scalding process, soil, dust, blood, feathers and intestinal content are released into the scald water and thus provide a significant opportunity for cross-contamination of carcasses, studies showed that the prevalence of *Campylobacter* generally decreased after scalding (Berrang and Dickens 2000; Guerin, Sir et al. 2010). *Campylobacter* have been isolated from the scald tanks (Stern, Fedorka-Cray et al. 2001; Klein, Reich et al. 2007; Reich, Atanassova et al. 2008) and the bacteria survival has been associated to scald temperature and water pH (Hudson and Mead 1987;

Humphrey and Lanning 1987; Berrang, Windham et al. 2011). The temperature of 52°C (soft scald), recorded for all samples collected in this study and normally used for carcasses that will be refrigerated using the air-chilling system has a lower effect on killing the pathogens, compared with the lethal effect of water at 60°C (hard scald) used for carcasses intended for water chilling (Reich, Atanassova et al. 2008). However, the continuous counter-flow system that exposes chicken carcasses to progressively cleaner water together with the introduction of fresh water and a final rinse at the exit of the scald tank prevent the accumulation of bacteria on chicken skin. The main issue with the scalding step is that this process can cause modifications to the superficial layer of the chicken skin, exposing crevices and feather follicles that can favour the attachment of the bacteria (Berndtson, Tivemo et al. 1992; Keum-Il, Min-Gon et al. 2007). Also during the following stage of de-feathering, entrapment of bacteria in skin crevices and feathers follicles can be facilitated by the action of the plucking machines. When entrapped within the chicken skin, the bacteria are more difficult to be removed by the subsequent final washing procedures (Berndtson, Tivemo et al. 1992; Keum-Il, Min-Gon et al. 2007). Bacteria trapped in the broiler skin also display greater resistance to the environmental factors during the processing (Yang, Li et al. 2001; Chantarapanont, Berrang et al. 2003; Nguyen, Fegan et al. 2012).

The de-feathering process is also considered a critical point for *Campylobacter* contamination, since the rubber fingers removing the feathers from the birds can become easily contaminated and may pass pathogens from bird to bird (Lee, Smith et al. 1998). In addition the automatic plucking machines can cause spilling of faecal contamination by pressing the rubber fingers against the abdominal cavity of the carcasses, and consequently increase the risk of *Campylobacter* contamination after

this step (Musgrove, Cason et al. 1997; Berrang and Dickens 2000; Berrang, Buhr et al. 2001; Guerin, Sir et al. 2010). Due to their design, these machines are also difficult to be cleaned properly at the end of the production shift, representing a further source for cross-contamination. Moreover, this step can cause spreading of microorganisms via aerosols as a result of the fingers rubbing the feathers off (Whyte, Collins et al. 2001; Allen, Bull et al. 2007). In the slaughterhouse investigated, requirements set in the EU Directive 852/2004 and EU Directive 853/2004 aimed to minimise air-borne cross-contamination, such as physical separation between the hanging-on bay, scalding/plucking and the evisceration area, were in place.

The purpose of the final washing is to remove contamination. Contrary to the findings reported in other studies (Guerin, Sir et al. 2010), a reduction of the level of *Campylobacter* contamination after final wash was not observed in this study. The final washing system consisted of a single inside/outside carcass washing cabinet, and it was considered as a critical control point in the HACCP plan for reducing any microbiological contamination. However, between the negative batches a significant increase on *Campylobacter* contamination was detected in carcasses collected after this step. This rise in the number of contaminated carcasses, and the increasing trend in the levels of contamination observed also among the positive batches and when all batches were included in the analysis, could be linked to the fact that due to line layout, the post final wash samples were collected immediately at the exit of the washing cabinet. At this point carcasses were still completely wet, with rinsing water still dripping from the neck skin at the moment of the sampling. Wempe et al. in 1983 reported that after the final washing, water remained on the chicken skin trapping the bacteria (Wempe, Genigeorgis et al. 1983). In addition, it has been shown that water

sprays used in the washing cabinets can create aerosols that could spread microbiological contamination (Berndtson, Danielsson-Tham et al. 1996).

In this study, the risk of cross-contamination of carcasses during the inside/outside carcass washing cabinet appeared to be more marked among the negative batches. Generally cross-contamination during carcasses washing occurs when sanitising treatments, such as chlorination of mains water supply, are not in place as an aid to improve hygiene during the operations (Berndtson, Danielsson-Tham et al. 1996; Bashor, Curtis et al. 2004). In our studied plant, chlorine dioxide was added to the main water supply according to the British Standards 12671: 2000. The final chlorine dioxide concentration in the water tank supplying water to the plant was 0.20ppm. The purpose of adding chlorine dioxide to the water supply system in the processing plant is to prevent cross-contamination of carcasses and equipment, and reduce microbial load by disrupting nutrient transport across the cell wall (FAO and WHO 2008). However, regardless of the pressure and of the volume of water used, the fact that the system was washing the carcasses with cold water did not lower water surface tension that is an important factor in bacteria/faecal removal (Keener, Bashor et al. 2004). Chlorine concentration higher than that allowed in potable water is not permitted in the EU but studies suggested that it is possible to achieve a reduction up to 2 log unit in *Campylobacter* numbers by using chlorinated water for the final carcass wash (Mead, Hudson et al. 1995; Bashor, Curtis et al. 2004).

As suggested above, there are numerous parameters that can affect the overall efficiency of the carcass washing system. Other parameters include numbers and types of washers, nozzles type, nozzle arrangement, length of the exposure of carcasses to water, line speed, and the use of sanitising agents. Bearing in mind the quantity of

water used for washing each carcass and the associated cost (Kiepper 2001), it is paramount that the machines are designed to make the best use possible of the water resources in the elimination of the contamination.

The removal of visually contaminated carcasses from the processing line (Boysen and Rosenquist 2009) and manual trim of the contaminated parts, would be an effective way to eliminate visual contamination. It is clear that the above solution would be difficult to implement, given the high speed of the processing line. However, it would be a most effective measure to remove the bacteria that are strongly attached to the carcass or entrapped within the skin, as it was shown that the final washing of carcass can only easily remove loosely attached bacteria. Furthermore, it has been demonstrated that a series of carcass washing at the various stages during the processing removes bacteria before they are retained, and this would be much more effective than a single final washing at the end of the slaughtering operations.

As reported in several studies, a decrease in *Campylobacter* positive carcasses associated with the chilling process was observed. Furthermore a decreasing trend in the bacteria counts was also observed after this step (Stern, Fedorka-Cray et al. 2001; Sánchez, Fluckey et al. 2002; Allen, Bull et al. 2007; Berrang, Meinersmann et al. 2008; Reich, Atanassova et al. 2008; Figueroa, Troncoso et al. 2009; Guerin, Sir et al. 2010). The purpose of chilling is to lower chicken carcass temperature to limit the growth of pathogenic and food spoilage microorganism and as a result to maintain a product that is safe for consumers. In the EU, to meet the safety requirements set in the Directive 853/2004, the carcasses have to be chilled to not more than 4°C as soon as possible after the slaughtering process. In the plant studied, the chilling was carried out through a dry air-chilling system, using cold air circulation. After the slaughtering

process that ended with the final washing as explained above, the carcasses were immediately moved into the chilling area. Compared to immersion chilling, air-chilling is the preferred chilling method used in Europe (James, Vincent et al. 2006). In this system, time of chilling, temperature and air speed are the main parameters that when combined can have different effects on the *Campylobacter* survival rate on meat products. However, they are mainly the drying conditions inside the chiller that are causing the higher physical stress to the bacteria (Klein, Reich et al. 2007; Figueroa, Troncoso et al. 2009), as it is well known that *Campylobacter* is highly susceptible to dehydration (Berrang and Northcutt 2005; Alter and Scherer 2006; Murphy, Carroll et al. 2006; Silva, Leite et al. 2011).

In our study the temperature inside the air-chiller was between -1°C to 1°C, and the chilling time was 75 minutes. At the exit of the chiller, the temperature of the carcasses, recognised as critical control point on the HACCP plan of the plant, ranged between 5°C to 10°C (critical control limit being set at <10°C). At the air-chiller exit, wings and legs were automatically separated from the rest of the carcass. Broiler carcasses were then weighed and transferred into a second chiller, where carcasses reached the legislative temperature requirement of below 4°C before entering into the breast meat portioning line. As our samples were collected after the first chilling step, a more dramatic reduction in the *Campylobacter* prevalence and bacteria count could be expected on carcasses after the second chilling stage, providing that no further contamination occurred in the meantime.

Interesting, among the negative batches, despite a reduction in the number of contaminated carcasses and in the level of contamination was observed moving from the final washing to the chilling step, the number of contaminated carcasses and the

level of contamination observed after the chilling process were higher compared to the initial level reported at evisceration. This shows that whereas for *Campylobacter*-positive batches the major risk of contamination of carcasses occurred at evisceration, the evisceration step appeared not to be the major risk of contamination of carcasses belonging to *Campylobacter*-negative batches.

When contamination on carcasses belonging to *Campylobacter* negative flock was reported from the first batch processed at the start of the production day, cross-contamination from the transport crates (Newell, Shreeve et al. 2001; Slader, Domingue et al. 2002; Hansson, Ederoth et al. 2005) and/or from the processing machines was identified as possible cause for the presence of *Campylobacter* on these carcasses (Kusumaningrum, Riboldi et al. 2003; Kudirkienėa, Bunevičienėa et al. 2011). Some studies could not detect *Campylobacter* on slaughtering machines after the cleaning and disinfection operations before the processing started (Miwa, Takegahara et al. 2003; Cools, Uyttendaele et al. 2005). However, Peyrat et al. in 2008 demonstrated that *Campylobacter* can survive through the cleaning and disinfection procedures (Peyrat, Soumet et al. 2008; Peyrat, Soumet et al. 2008). Pre-existing biofilms formed by other bacteria may enhance the attachment of *Campylobacter* on these surfaces (Hanning, Jarquin et al. 2008) and within biofilms the bacteria could be protected from the sanitising procedures increasing the bacteria survival in the slaughterhouse environment (Newell, Shreeve et al. 2001; Chmielewski and Frank 2003; Peyrat, Soumet et al. 2008; Kudirkienėa, Bunevičienėa et al. 2011; Nguyen, Fegan et al. 2012).

The fact that cross-contaminated carcasses, belonging to negative batches and found at the start of the production day, were mostly observed post evisceration and after

final washing, could be explained by the fact that bacteria that survived throughout the night cleaning and sanitising process were stressed (Peyrat, Soumet et al. 2008). Indeed it appeared that these carcasses were mainly contaminated with more fragile bacteria, loosely attached to the carcasses that did not survive to the subsequent chilling stage (Newell, McBride et al. 1985). More in depth research is required to better understand the mechanisms associated with the survival of *Campylobacter* cells in the slaughterhouse environment and to identify the appropriate sanitising practices needed for the complete elimination of the bacteria at the end of the production day.

Despite the numerous sources for carcass contamination and the various steps in which the bacteria can spread during the processing, our study reported that it is also possible for broiler flocks to remain *Campylobacter*-free from farm to finished product. Indeed on Sampling Day 4, only chickens from one farm were processed, and they were all found to be *Campylobacter* negative at caeca and carcass level. This brings to light the importance of the incoming flocks on the level of *Campylobacter* in the slaughterhouse environment and in the successive contamination of the final products.

In conclusion during the slaughtering process, bacterial levels on the carcasses may either increase or decrease throughout the various steps of the process. During the processing effective production layout, efficient machines design, flock uniformity, stoppage of faecal leakage, elimination of any unnecessary contact of carcass with potential contaminated surfaces and high-quality training of personnel in food hygiene, are crucial measures to produce safe meat products. However, if at the source of poultry production, no adequate measures are in place to minimise the flock colonisation, all the efforts and the hygienic practices of the processing plant may not be sufficient to deliver safe products to the final consumer.

CHAPTER 4

CAMPYLOBACTER IN TWO DIFFERENT BROILER COMMERCIAL SYSTEMS FROM FARM TO FORK

4.1. INTRODUCTION

Campylobacter is one of the main causes of human bacterial gastroenteritis in the world. Since 2005 the EU notification rate of human campylobacteriosis has followed an increasing trend. In 2011, campylobacteriosis was, once again, by far the most frequently reported zoonotic disease in humans (EFSA 2013). Also in the UK, since 2004, the number of reported cases has been rising each year (HPA 2012) and with over half a million people infected every year (Tam, Rodrigues et al. 2012) *Campylobacter* is now the most frequent cause of food poisoning in the UK (FSA 2013). For some people, particularly the elderly population over 70 years of age, this can be a serious infection: on a yearly basis around 20,000 people are admitted to hospital for treatment and alarmingly about 110 people die in the UK (FSA 2013; Humphrey 2013). The sources of human infections are multiple and varied. However, epidemiological studies have shown that handling and consumption of poultry is the most important source of human campylobacteriosis (EFSA 2010d).

It is well known that poultry flocks become colonised with *Campylobacter* during the rearing period following exposure to viable bacteria from the environment, and *Campylobacter* in the caecal contents can be detected after only a few hours (Bull, Allen et al. 2006). There are multiple potential routes of introduction of *Campylobacter* into a commercial broiler flock. Poor hygiene standards, unsatisfactory farm management together with inadequate bio-security practices, farming of other livestock other than chickens around the farms, presence of pests and lack of vermin control, use of untreated drinking water, short intervals between flocks, thinning practices, presence of flocks of different ages, seasonality and husbandry methods

have all been previously reported as risk factors for the introduction of *Campylobacter* into the broiler flock (Berndtson, Emanuelsonb et al. 1996; Hald, Wedderkopp et al. 2000; Cogan, Slader et al. 2002; Newell and Fearnley 2003; Bouwknegt, van de Giessen et al. 2004; Zweifel, Scheu et al. 2008; Ridley, Morris et al. 2011; Bahrndorff, Rangstrup-Christensen et al. 2013). Several studies have shown that in colonised chickens, the numbers of this pathogen may be as high as 10^9 cfu per gram of caecal contents (El-Shibiny, Connerton et al. 2005). Furthermore during processing, the bacteria are spread into the poultry meat products and are able to survive throughout the food chain until the products reach the final consumer.

This epidemiological study was undertaken during one year to investigate the *Campylobacter* prevalence in two housed commercial production systems. The aim of the work presented in this chapter was to investigate the potential impact of each production system on the incidence of colonisation in broiler flocks during the whole rearing period. To achieve the above, it was necessary to single out which specific factors related to the different production systems influenced the *Campylobacter* colonisation and the subsequent contamination of the final products.

Given that the presence of this pathogen in the meat products constitutes the major risk for consumer exposure, it is essential to determine the prevalence of *Campylobacter* at the end of the food chain. Therefore, in this study, breast meat fillets were collected at the end of processing, to estimate the actual risk that the final chicken meat product is posing to human health. In summary the objective of this study was to link the flock *Campylobacter* status to the risk of contamination on the consumer's plate.

4.2. MATERIALS AND METHODS

4.2.1. *Study design*

The impact of the production system on *Campylobacter* prevalence and incidence was investigated in two different commercial production systems (Standard system and Freedom Food Indoor) previously described in Chapter 2. The production systems were chosen within one integrated poultry company, and they differed mainly in breed used and stocking density. Other differences between the two systems included lighting requirements, environmental enrichment, growth rate and feed type. The characteristics of the two production systems are summarised in the Table 4.1 below.

	Standard System (ST)	**Freedom Food Indoor (FI)**
Standard	ACP (Red Tractor)	RSPCA
Breed	Ross 308	Hubbard JA57
Sex of Birds	Birds sexed	As hatched
Stocking density (kg/m²)	38	30
Housing & environment	Standard	Standard with windows
Environmental enrichment	No	Straw bales perches and pecking objects
Range requirement	Indoor	Indoor
Thinning requirement	Practiced	Full depopulation min 49 days old
Stunning Requirements	No set standard, however birds must be rendered unconscious.	Stun to kill

Table 4.1: Summary of the production systems' features

In order to establish the level of *Campylobacter* colonisation from farm to fork in the two production systems investigated, a total of four Standard farms and four Freedom Food farms were recruited and sampled throughout a 6–8 weeks production cycle. For each farm, 3 distinctive production cycles were investigated at different times of the year to take into consideration the seasonality of this pathogen.

All 24 flocks were examined for the presence of *Campylobacter* at the farm level during the whole rearing period, and during the slaughtering/cutting and packaging operations in the processing plant. In the farm, during the rearing period, each flock was visited 3 times. Information on management factors potentially influencing flock colonisation was collected during the first visit using a questionnaire and through personal interview with the farm manager. The questionnaire was focused on bio-security practices at the farm gate, policy for visitors, hygiene facilities, use of protective clothing, water quality, staff awareness of bio-security, control of pests and wild birds, presence of other livestock, waste control, catching operations and final cleaning (Appendix 6).

4.2.2. Data collection, sampling procedures and aims of sampling

The unit of analysis was the 'flock', defined as a group of chickens placed in the farm at the same time and raised in the same broiler house. Three farm visits were carried out per production cycle for each flock.

On the day of chick placement (Day 1), five papers lining the cardboard crates used to transport the birds from the hatchery to the farm (100 chicks per crate) were collected to examine the *Campylobacter* status of the incoming chicks. The lining papers were folded into a clean plastic bag and transported to the laboratory.

The second farm visit was carried out halfway through the flock production cycle. Ten healthy birds were collected during the farm visit. Birds were chosen ad hoc in different areas of the study house, and were euthanized by neck dislocation. The carcasses were immediately transported to the laboratory and a post mortem examination was carried out. From each animal, caecal samples were analysed individually to assess the load of flock colonisation at that particular stage.

The last farm visit was carried out the day before the first depopulation of the flock. Ten healthy birds were collected to establish flock colonisation and the baseline level before the beginning of the catching and transport, to investigate any potential increase in the *Campylobacter* count due to the stress undergone by the birds during these operations (Knowles and Broom 1990; Nicol and Scott 1990; Mitchella and Kettlewella 1994; Mulder 1995; Whyte, Collins et al. 2001).

From each studied flock, on the day of the first depopulation, one batch of birds delivered to slaughter in the same vehicle was sampled during processing. All those batches were slaughtered and processed in Plant A, as previously described in Chapter 2. At slaughter, from each batch, 10 caecal samples were collected at the time of carcass evisceration by manual traction at the junction with the intestine to estimate *Campylobacter* flock colonisation. In order to assess carcass contamination during processing, 10 neck skin samples were collected at the end of the slaughter line

directly after the air-chiller and before any further processing such as cutting or packaging. Only intact caeca were sampled, and neck skin samples were collected from carcasses free from any visible contamination. After collection, samples were kept individually in a sterile bag, under refrigeration conditions until processing. Samples were processed within 2 to 24 hours of collection.

At slaughter, flock information was collected from the Food Chain Information (FCI) made available for each batch sampled by the Food Business Operator (FBO) (Appendix 1). During the sampling, flock welfare parameters such as pododermatitis, hock marks, total and partial rejection of products declared unfit to enter into the food chain were assessed and figures recorded. Processing data such as batch catching time, time of arrival on site, time of processing, killing order, line speed, temperature of the water in the scald tank, temperature inside the air-chiller, time of air-chilling process, carcasses temperature at the exit of the air-chiller and concentration of chlorine dioxide in the water supply were recorded. Furthermore, slaughter operations, hygiene standards and visible carcasses contamination were evaluated during the processing. A questionnaire, including flock and slaughterhouse information, was completed for each batch sampled (Appendix 2).

Twenty four to 48 hours after slaughter, 2 to 5 packages of breast meat fillets ready to leave the premises for the supermarket, were collected from each flock to identify the bacterial load in the final products. Breast meat packages were kept under refrigeration conditions until microbiological analysis.

For all Standard flocks, one extra sampling at slaughter was carried out in a second processing plant (slaughterhouse D) around 10 days after the first depopulation event.

No major differences were observed in slaughterhouse D compared to slaughterhouse A, other than slaughterhouse D was adapted to receive and process birds which were double the size of the broilers processed in slaughterhouse A. This extra sampling was not carried out in the Freedom Food flocks because, in these flocks, the depopulation was done in a single day, as according to the Freedom Food guidelines the partial depopulation of the flock was not recommended (RSPCA 2011). Table 4.2 summarise at which level data were collected.

Data collected at farm level	Data collected at flock level	Data collected at batch level	Data collected on individual birds
Breed	Stocking density	Age at slaughter	*Campylobacter* count
Feed Mill	House mortality	Weight at slaughter	
Flock size	Disease history	Rejection at slaughter	
Number of houses	Diseases diagnosed	Pododermatitis	
Total m^2	Medications prescribed	Hock burns	

Table 4.2: Level at which data were collected

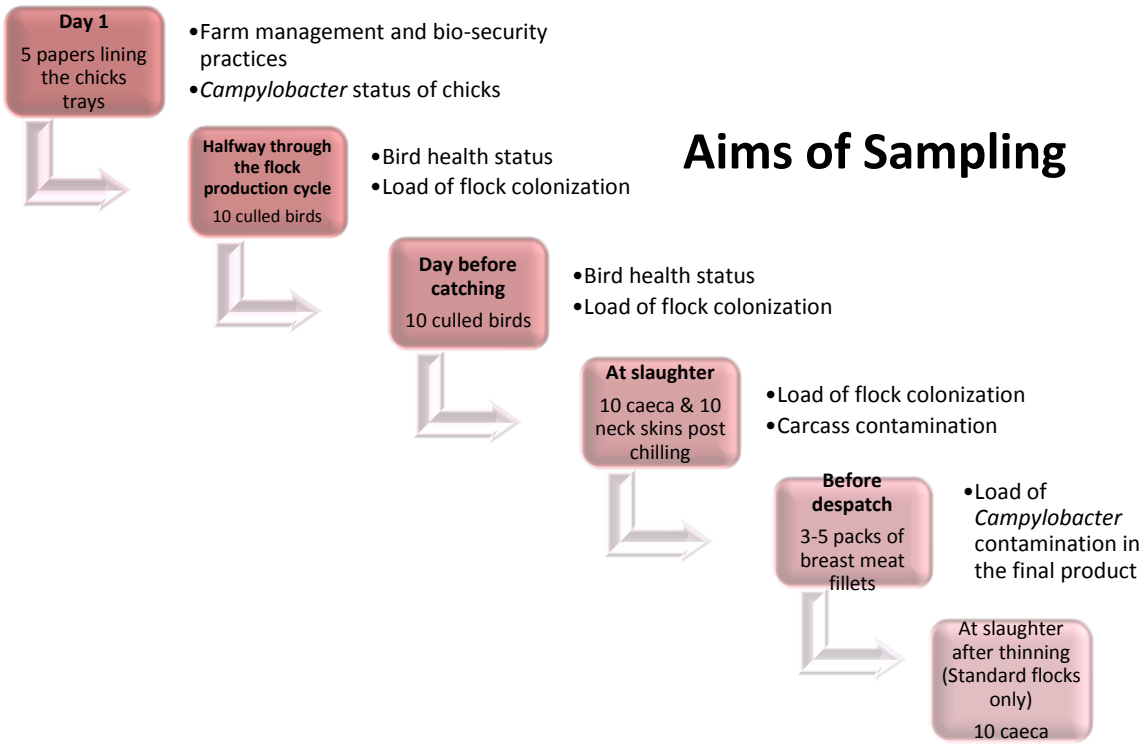

Figure 4.1: Stages at which the samples were collected, types of samples collected and aims of sampling

4.2.3. Campylobacter detection and enumeration

4.2.3.1. *Campylobacter detection on papers lining the chicks' transport crates*

Each paper lining the chick transport crates was analysed individually. A cotton swab (MWE Medical Wire) was pre-moistened in MRD and used to swab different soiled areas of the paper. The swab was then directly plated on mCCDA and the plate was incubated under microaerobic conditions (MACS-VA 1000- microaerobic workstation: Nitrogen 80%, Hydrogen 3%, Oxygen 5%, C. Dioxide 12%) at 41.5°C for at least 48 hours.

A second swab pre-moistened in Bolton broth prepared in house (*Campylobacter* enrichment broth, Lab M, with lysed horse blood (1%), and CVTN supplement- cefoperazone, vancomycin, trimethoprim, cycloheximide- Lab M) was used on different soiled areas of the paper and then was inoculated into a sterile container containing 3ml of Bolton broth. After 24 and 48 hours of incubation in microaerobic conditions, one loop-full of broth was streaked out on mCCDA plate and the plate was incubated under microaerobic conditions at 41.5°C for a further 48 hours. Using a third swab, the same enrichment step was followed with modified Exeter broth (*Campylobacter* enrichment broth, Lab M, with lysed horse blood (1%), and *Campylobacter* growth selectavial SV59-trimethoprim, rifampicin, polymysin B,Cefoperazone, amphotericin- MAST Diagnostic). After 48 hours of incubation, all the plates were examined for the presence of *Campylobacter* presumptive colonies.

4.2.3.2. *Enumeration of Campylobacter in caecal and neck skin samples*

For caecal and neck skin samples, methods for *Campylobacter* enumeration were based on the ISO method 10272-2:2006 as aforementioned in Chapter 2 (ISO 2006).

4.2.3.3. *Campylobacter detection and quantification techniques on breast meat*

Depending on the number of the breast fillets contained in each package, two to four skinless breast meat fillets were analysed individually. From each package, at least one fillet was analysed for superficial contamination and one for deep muscle contamination.

4.2.3.3.1. Superficial contamination analysis

Wearing disposable gloves, a chilled breast meat fillet was removed from the retail package taking care not to contaminate the fillet's outer surface. Using sterile instruments and aseptic techniques, a section of 25 grams of breast meat surface was removed. The rest of the fillet was put into a stomacher bag containing 100ml of MRD and it was rinsed by agitating and shaking the bag manually ensuring that the MRD came into contact with all surfaces of the fillet. After one minute, the fillet was removed and discarded from the bag and the test portion of 25 grams previously prepared was transferred to the rinse solution. The mixture (1 in 4) was treated in a stomacher (Micro-Biomaster, Seward Stomacher 80, Lab System) for one minute at normal speed. 125ml of MRD was added subsequently in order to adjust to a 1 in 10

solution. From the suspension (10^{-1}) 100µl was plated through spread plating onto a mCCDA plate.

4.2.3.3.2. Deep muscle contamination analysis

To examine deep muscle contamination the second fillet from the same breast meat package was used. A sterilisation step was used to ensure that the surface of the fillet was free of any *Campylobacter* contamination. In the first part of the study, that included all the breast meat samples collected from the flocks processed during the first production cycle, the breast meat surface was sterilised using a hot spatula repeatedly flamed by means of a Bunsen burner. In the second part of the study, that included all the breast meat samples collected during the second and third production cycles, the surface of the fillet was sterilised using an electric wok: the inner fillet from the breast fillet was removed to smooth the surface, and then the whole fillet surface was put in contact with the heat (Figure 4.2). Each fillet was kept in the wok for no longer than 2 minutes, as it appeared that the bacteria on the surface could be destroyed easily by the limited cooking as they are sensitive to heat treatments (Silva, Leite et al. 2011). However, the short 'cooking' time was not enough to kill any potential *Campylobacter* contamination in the deep muscle (Blankenship and Craven 1982; FAO and WHO 2002; de Jong, van Asselt et al. 2012).

Figure 4.2: Sterilisation step of the surface of the fillet

To verify the effectiveness of the sterilisation procedure, after removing the sample from the wok, the whole fillet was rinsed with 10ml of MRD and then one loop-full of the rinse was direct plated on three mCCDA plates, on three CAB plates and on three NA plates (Nutrient agar, Lab M, LAB008). Each distinct agar plate was then incubated under microaerobic conditions at 41.5°C, aerobic conditions at 37°C and in aerobic conditions at 30°C for at least 48 hours.

After the sterilisation of the surface, using sterile instruments and aseptic techniques, the upper layer of the fillet was removed (Figure 4.3), and 25 grams of deep tissue was transferred to a stomacher bag and 100ml of MRD was added. The mixture (1 in 4) was treated in the stomacher for one minute at normal speed. 125ml of MRD was added subsequently in order to adjust to a 1 in 10 solution. From the suspension (10^{-1}), 100µl was plated through spread plating techniques on mCCDA.

Figure 4.3: Investigation for deep muscle contamination:
deep muscle exposed after sterilisation and ready for sampling

All plates were then incubated under microaerobic conditions (MACS-VA 1000-microaerobic workstation: Nitrogen 80%, Hydrogen 3%, Oxygen 5%, C. Dioxide 12%) at 41.5°C for at least 48 hours. After incubation, plates were examined and numbers of *Campylobacter* presumptive colonies were counted in order to calculate the number of these bacteria per unit of sample. Final results were recorded as colony forming units per gram of breast meat (cfu/g). The detection limit of this protocol, according to the dilution factor used, was 100 cfu/g.

From all samples, presumptive *Campylobacter* colonies were identified directly from mCCDA plates by their typical appearance (grey, flat, irregular and spreading colonies) by microscopic examination at Gram Stain (Gram negative curved rods) and an inability to grow under aerobic conditions (HPA 2011). From each *Campylobacter* positive sample, up to 5 presumptive colonies of different morphologies were retained for later typing. Isolates were confirmed and identified to the species level *(C. jejuni or C. coli)*

using two distinct PCR assays described in Chapter 5 (Linton, Owen et al. 1996; Klena, Parker et al. 2004; Katzav, Isohanni et al. 2008).

4.2.3.3.3. Enrichment step

Compared to the *Campylobacter* detection procedures followed for caecal and neck skin samples, where direct plating was the only method used; for the bacteria detection in breast meat fillets selective enrichment broths for *Campylobacter* recovery were also used. The enrichment step was included to improve detection where *Campylobacter* may be at low numbers, injured and/or stressed and where also low background floras were expected (Beuchat 1986; Corry, Post et al. 1995; Mason, Humphrey et al. 1999; Abulreesh, Paget et al. 2005). For each breast meat sample, 1ml of the initial suspension (10^{-1}) was inoculated into a sterile container containing 3ml of Bolton broth and in a second sterile container containing 3ml of Exeter broth. After 24 and 48 hours of incubation under microaerobic conditions, from each container one loop-full of broth was inoculated on mCCDA plates and the plates were incubated under microaerobic conditions at 41.5°C for a further 48 hours.

4.2.4. Data management and data analysis

Logarithmic transformation was applied to all welfare indicator's variables: house mortalitity, rejections, pododermatitis and hock burn and Student t-tests were used to examine differences in welfare indicators among the production systems. Chi-squared $\chi 2$ test was used to investigate the association between welfare indicators and *Campylobacter* colonisation. A p value of 0.05 was considered significant. Confidence

intervals of all welfare variables were calculated considering the clustering of birds within the farms.

All colony-forming units results were transformed to $\log_{10}$ counts per gram before statistical analysis. Samples in which *Campylobacter* was detected below the enumeration limit were assigned to the value 1 to allow the calculation of the $\log_{10}$ value. The distribution of the enumeration values was highly skewed also after the log transformation and non-parametric statistical analysis was conducted. *Campylobacter* prevalence between the two production systems and the association between batch colonisation and carcass contamination was investigated using chi-squared $\chi 2$. Similarly, 2-sample Wilcoxon rank-sum (Mann-Whitney) test was used to compare microbial counts. Confidence intervals for *Campylobacter* counts were calculated considering the clustering of birds within the farm. A p value of 0.05 was considered significant.

Except when confidence intervals are provided for an estimate of welfare parameters and *Campylobacter* counts, the analyses carried out in this study have not considered the hierarchical structure of the data (bird>batch>farm) and the clustering of birds within batch/farm/slaughterhouse. The statistical analysis was carried out using Stata version 9 (StataCorp 2005)

4.2.5. Ethical considerations

The research project was approved by the University Committee on Research Ethics. A consent form from the all the farms involved in the project was requested before the commencement of the study (Appendix 4).

4.3. RESULTS

This one-year epidemiological study was undertaken to examine the *Campylobacter* prevalence in two housed commercial broiler production systems, to investigate farm and slaughter factors linked to the different production systems that could influence *Campylobacter* colonisation and carcasses contamination.

4.3.1. Farm descriptions

The 8 farms involved in this study (4 Standard and 4 Freedom Food Indoor farms) were randomly chosen from a list provided by a large UK poultry producer (Table 4.2). All farms were located at different geographical locations in North Wales and the Northwest of England (Figure 4.4).

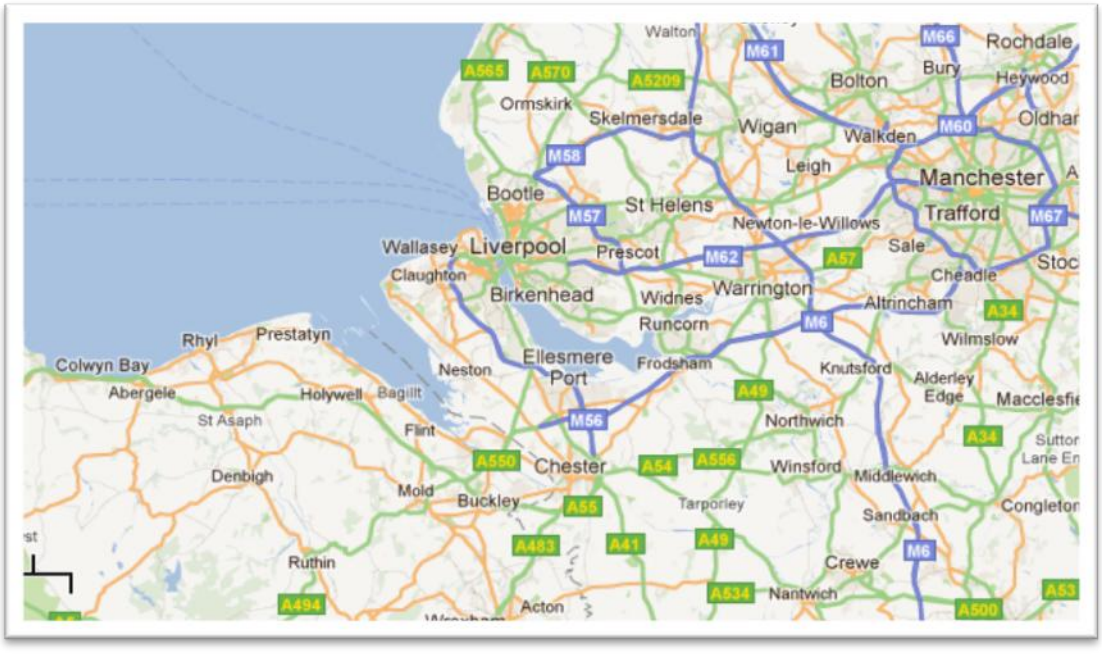

Figure 4.4: Geographical area where farms were located

All contacted farmers consented to be part of the study. All the farms were relatively small, with 3 or 4 broiler houses. The houses were closed, insulated and with regulated temperature and ventilation. They were equipped with automatic feeding and drinking systems. Drinking water for the birds was obtained from disinfected water from an intermediate storage tank filled by the mains water supply. For each farm, a target house was selected for the sampling, and the same target house was used for the duration of the study.

Only three farms (all Standard ones) were owned by the integrated poultry producer. In those farms the same bio-security practices were observed. The first hygiene barrier was located at the entry of the farm premises where there was a designated visitors' car park. At this point, visitors entering the farm were required to wear protective clothing (overalls, boots and masks). Boots were provided on site. A second hygiene barrier was located in the anteroom of each broiler house; it included hand-washing and sanitisation facilities and required a change of boots. Furthermore, before entering into the flock the boots were sanitised using foot dips. In these farms, the 'all in all out' policy was applied to the entire farm, as the chicks' placement was carried out the same day in all the broiler houses. During the farm visits, no obvious differences were identified in terms of farm management, design of buildings and general tidiness in all three company farms.

The rest of the farms (one Standard and four Freedom Food farms) were tied to the integrated poultry producer by long-term contracts that included the supply of day-old chicks and feed, and management supervision. No hygiene barrier at the farm gate was in place in these farms. The first hygiene barrier was located directly at the entry

of the broiler houses, which required the wearing of protective clothing (overalls, boots and masks). Boots were not provided on site, and disposable ones were used for each visit by the visiting researcher. Changing of footwear was not requested when entering into the broiler flock. However, boots were sanitised through the use of foot dips placed at the entry of the broiler house and in the anteroom.

Two farms selected for this study (Farm 4 and Farm 5) belonged to the same family and were located in the same farm yard. Farm 4 had two houses which produced Standard birds. Farm 5 had two newer houses which belonged to the Freedom Food scheme.

Two more Freedom Food farms (Farm 2 and Farm 7) selected in this study belonged to the same family business. The two sites were 2 miles apart and they were run and managed by the same staff. The staff did not change working clothes when moving from one site to the other. According to commercial requests by the integrated poultry company, both farms reared at the same time Freedom Food flocks together with Standard flocks in different broiler houses. This meant that the whole farms were never empty, as chicks belonging to the different production systems were placed on the farms at different times, according to the poultry company's demand.

The last contract Freedom Food farm that was studied (Farm 8), belonged to a small poultry business that had 2 more poultry farms located in the Northern part of the UK, which raised Standard birds. Different members of staff worked on the three different poultry sites. However, the farm management was the same in all three farms and they made regular visits between the different sites.

In all investigated farms, DEFRA approved disinfectants were used in the foot dip facilities. However the brands of these chemicals used were different in all farms. Farm staff was monitored during the visits and were always observed cleaning their boots by immersing them into the foot dip facilities before entering into the poultry flock. Despite this, no systems were in place to verify the concentration of the disinfectants used. Furthermore the water in the foot dip was not changed systematically, but it was changed only when considered dirty by the farm manager.

In all four Standard farms, female and male birds were kept separated inside the house. Female birds (pullets) were sent to slaughter at around 2.21 kg (95% CI 2.19-2.23) at around 39 days (95% CI 38.70-39.27). At that point, in the farms the fences inside the sheds were removed and male chickens (cockerels) were allowed to take up all the space in the sheds, reaching an average weight per bird of more than 4kg at slaughter which occurred 10-12 days after the first depopulation (at around 51 days, 95% CI 50.91-51.42). The above thinning practice, did not happen in any of the Freedom Food farms, as partial depopulation of the flock is not advisable by their internal regulation (RSPCA 2011) and female and male chickens were not kept segregated inside the broilers in any of the Freedom Food farms investigated.

In all 8 farms selected, birds were kept on litter which mainly consisted of wood shavings and the birds had free access to water and feed. At the end of each production cycle, all litter was completely removed and the broiler house was thoroughly cleaned and disinfected. The house was then kept empty to dry, and the downtime between flocks ranged between 1-2 weeks in all investigated farms. In all company farms at the end of each production cycle, 10 swabs per farm were taken and

sent to the company laboratory to verify the final cleaning and disinfection procedures. However, microbiological results were not available on site. All farms operated a rodent control programme and no other livestock farms were adjacent to the studied farms.

Farm ID	Farm Code	C/P	Type of Production	No. Houses	Total m^2	Total birds	Stocking Density	Breed
1	Long ST1	C	Standard	4	6420	106400	38	Ross 308
2	Long FB1	P	Freedom Food	3	3012	50100	30	Hubbard JA57
3	Long ST2	P	Standard	3	4815	79800	38	Ross 308
4	Long ST3	C	Standard	2	6075	100900	38	Ross 308
5	Long FB2	C	Freedom Food	2	6075	100900	30	Hubbard JA57
6	Long ST4	P	Standard	3	4815	79800	38	Ross 308
7	Long FB3	C	Freedom Food	4	6738	54800	30	Hubbard JA57
8	Long FB4	C	Freedom Food	3	4815	79800	30	Hubbard JA57

Table 4.3: Farms summary (C=Contract farm, P=Company farm)

4.3.2. Flock colonisation during the rearing period

The first production cycle for all 8 investigated flocks, was screened between August and November 2011. The second and the third crops were screened between January and April 2012 and between March and July 2012 respectively.

As reported in several other studies, *Campylobacter* was not cultured from the paper liners collected on the day of the chicks placement, either by direct plating or after the

enrichment steps (Berndtson, Danielsson-Tham et al. 1996; Herman, Heyndrickx et al. 2003).

In both production systems, apart from day one, *Campylobacter* was isolated at all stages during the rearing period. Once flock colonisation was confirmed, all the caecal samples were positive. In contrast to other studies, (Allen, Bull et al. 2007; Hansson, Forshell et al. 2007; Jorgensen, Ellis-Iversen et al. 2011) no low prevalence of *Campylobacter* in the flocks was observed at any stage during the rearing period in both production systems.

As reported in other studies, as the flocks aged more became positive across the 24 crops (Herman, Heyndrickx et al. 2003). Halfway through the production cycle, only 25% of the flocks were found to be colonised (6 out of 24). At slaughter, at first depopulation, 54% of flocks were *Campylobacter* positive (13 out of 24). After the first thinning, 100% *Campylobacter* colonisation was observed among the Standard flocks at slaughter. This increasing trend was observed in both production systems (Figure 4.5).

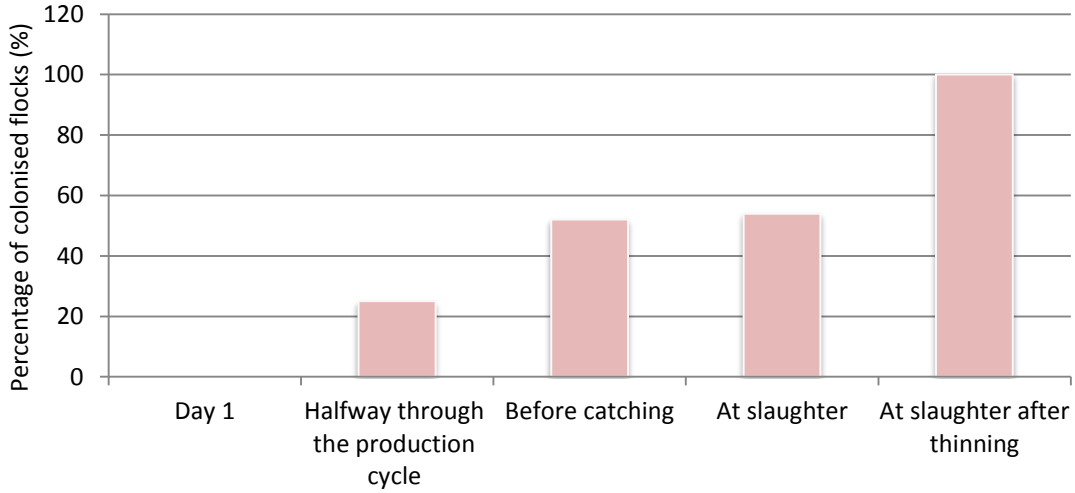

Figure 4.5: Percentage of colonised flocks during the rearing period

4.3.2.1. Campylobacter and catching/transport operations

On the day of the slaughter (first depopulation), birds were loaded into plastic crates by the catching team. Crates were stocked with 15-25 birds according to bird weight, weather conditions and farm size. The length of time spent in transport was similar for all the investigated flocks as the slaughterhouse was located within a 40 miles radius of the farms. Among the colonised flocks, the transport time varied between 1.5 hours and 2 hours. It was the slaughterhouse internal welfare policy to keep animals waiting on site to a minimum. However, due to line breakdown, occasionally longer holding times in the plant occurred. Among the colonised flocks the holding times varied between 35 minutes and 2.5 hours. According to transport time and holding time, the total time that a colonised flock spent inside the crates ranged between 2.5 hours and 4.33 hours. To investigate if the total time spent in the crates had an impact on the *Campylobacter* numbers in caecal content, flocks were divided in 3 groups according to the time spent inside the crates. In the first group animals spent less than three hours, in the second group less than four hours and in the last group less than five hours. All flocks identified as *Campylobacter*-free before catching remained so following transportation. In both production systems, a significantly higher average *Campylobacter* count was observed in caecal samples after transport (2-sample Wilcoxon rank-sum- Mann-Whitney; $p<0.001$). This increase was independent of the total time spent by the animals inside the crates.

Within the individual colonised flocks an increase in the bacterial counts was observed in all flocks after transport. Two-sample Wilcoxon rank-sum test-Mann-Whitney showed statistically significant higher counts after transport for the 42% of the

colonised flocks. A significant increase in the level of *Campylobacter* after transport occurred in 67% (4 out of 6) of the colonised Freedom food flocks and in only one colonised Standard flock (1 out of 6). In one of the Standard flock positive at slaughter (Farm 1, production cycle number 3), the sampling before the catching was missed due to a misunderstanding with the farm management.

4.3.2.2. Flock colonisation at slaughter

At slaughter, during the first depopulation event, out of the 24 investigated flocks, 13 (54%) were *Campylobacter* positive. A higher *Campylobacter* prevalence (7 out of 12, 58%) was reported among the Standard production system compared to the Freedom Food Indoor (6 out of 12, 50%) (Table 4.4). However, a statistically significant association between flock colonisation and production system was not observed in this study (Chi-squared $\chi 2 = 1.678$; $p=0.195$). Quantification analysis showed a high level of *Campylobacter* within all the positive flocks. Although high counts were reported in both production systems, the average *Campylobacter* count was significantly higher in the Standard population (Two-sample Wilcoxon rank-sum (Mann-Whitney) test $z=2.916$, $p<0.01$) (Table 4.4).

Production system	**Flock sampled**	**Flock positive (%)**	***Campylobacter* mean log cfu/g**	
			Mean	**95% Conf. Interval**
Standard system	12	7 (58)	4.838	3.237 - 6.439
Freedom Food system	12	6 (50)	3.850	-0.796 - 8.495
Total	24	13 (54)	4.342	1.762 – 6.921

Table 4.4: Flock colonisation prevalence and Campylobacter count at slaughter (first depopulation event) by production system including 95% CIs calculated with farm as cluster factor

More flocks were colonised at slaughter during the autumn season, compared to flocks sampled throughout the rest of the year ($p<0.01$). Furthermore *Campylobacter* counts were not associated with production cycle and season at slaughter.

Campylobacter counts on caecal samples appeared instead to be related to flock colonisation at half way through the production cycle. Among the *Campylobacter* positive flocks at slaughter, a significantly higher count in caecal samples was observed when flocks were found positive at half way through the rearing period ($p<0.05$). This was observed in both production systems.

During the second depopulation event, among the *Campylobacter* positive Standard flocks, only one had higher count after the first thinning. In the rest of the flocks we observed a decrease in the *Campylobacter* counts after the first depopulation event. For three flocks this reduction was statistically significant ($p<0.05$).

4.3.3. Flock colonisation and welfare parameters

Birds from the Standard flocks grew more rapidly compared to birds belonging to the Freedom Food scheme. In both production systems, at the first depopulation event, birds reached the same market weight of just over 2kg (95% CI 2.060 – 2.256) at 39.983 days (95% CI 38.461 - 39.505) in the Standard flocks, and approximately at 49.244 days (95% CI 48.953 - 49.533) in the Freedom Food flocks. At the time of the first depopulation event, significant differences were observed between the two production systems in the prevalence of house mortality (t-test $_{df=187}$ = 11.159;

p<0.0001), rejections (t-test $_{df=207}$ = 4.907; p<0.0001), pododermatitis (t-test $_{df=207}$ = 17.153, p<0.0001) and hock marks (t-test $_{df=207}$ = 19.451; p<0.0001) (Figure 4.6).

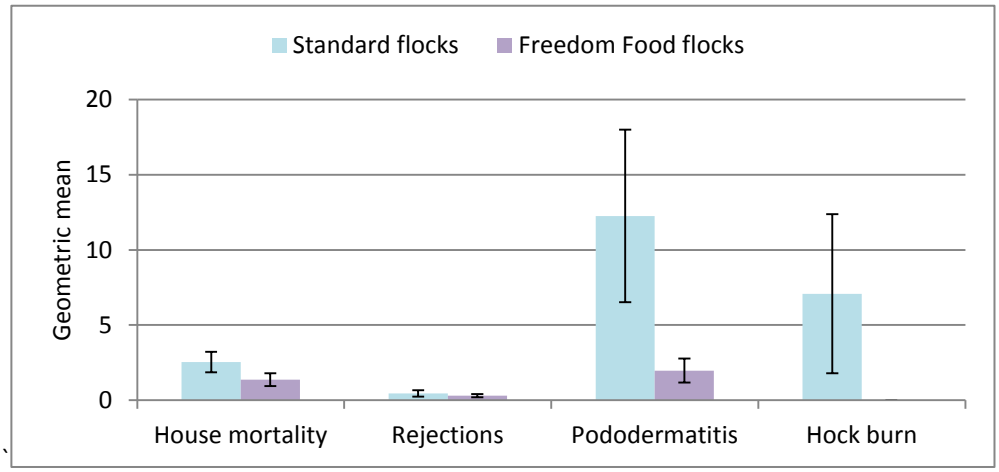

Figure 4.6: Welfare indicators by production system at the time of first depopulation event including 95% CIs farm as cluster factor

An important association of *Campylobacter* colonisation and welfare parameters were seen between the flocks investigated. In *Campylobacter*-free flocks levels of house mortality (t-test $_{df=187}$ =-5.1016; p<0.0001), rejections (t-test $_{df=207}$ =-1.8584; p=0.0645), pododermatitis (t-test $_{df=207}$ = -1.1906; p=0.235) and hock burns (t-test $_{df= 207}$ = -4.5956; p<0.0001) were lower compared to colonised flocks. However, when the clustering of the birds within farms were taken in consideration and the analyses was carried out by individual breed, *Campylobacter* infection appeared associated only with hock burns among the faster growing breed (Figure 4.7).

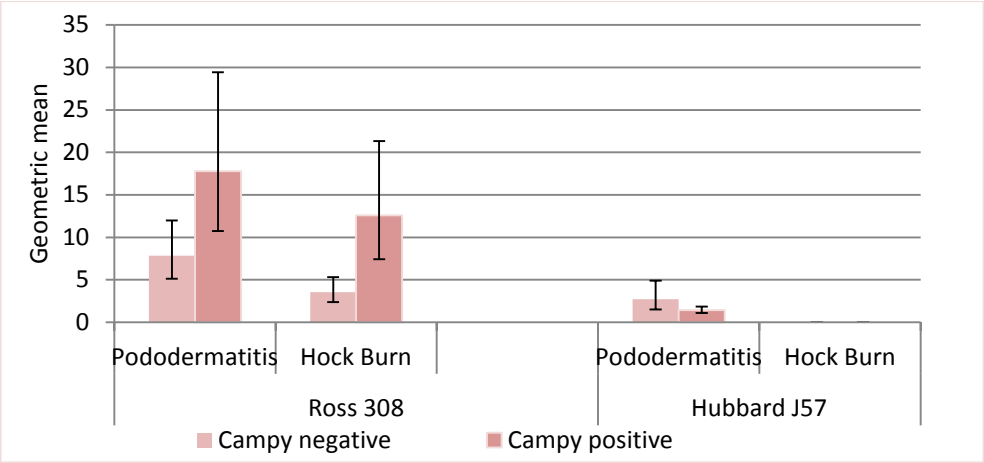

Figure 4.7: Percentage of pododermatitis and hock burns by campylobacter colonisation status and by breed including 95% CIs calculated with farm as cluster factor

4.3.4. Carcass contamination

The number of carcasses identified as *Campylobacter* positive post air chilling was 113 out of 220 (51%). A significant association between carcass contamination and production system was not observed. However, a significant association was reported between carcass contamination and flock colonisation at slaughter (first depopulation) (Chi-squared $\chi 2=178.8294$; $p<0.001$). Among the 120 neck skin samples collected from *Campylobacter* positive flocks, 111 samples (92%) were contaminated. Among the 100 carcasses collected from *Campylobacter* negative flocks only 2 (2%) were positive for *Campylobacter*.

The average number of *Campylobacter* on positive carcasses from slaughter groups with negative caecal samples was significantly lower (0.056 log cfu/g) than the average count (2.992 log cfu/g) reported on carcasses belonging to *Campylobacter* positive batches (Two-sample Wilcoxon rank sum (Mann-Whitney) test $z=-12.388$; $p<0.0001$).

Enumeration results on carcasses showed significantly higher average *Campylobacter* counts among the Standard system compared to the ones belonging to the Freedom Food system (Two-sample Wilcoxon rank-sum (Mann-Whitney) test z=3.852; p<0.0001).

The table below (Table 4.5) summarises the *Campylobacter* counts detected on broiler carcasses in the two different production systems considering the clustering of birds within farms.

Production System	Mean log cfu/g	95% Conf. Interval
Standard System	2.082	1.815 – 2.349
Freedom Food Indoor System	1.193	0.527 - 1.859

Table 4.5: Campylobacter mean log cfu/g in broiler carcasses among the different production systems including 95% CIs calculated with farm as cluster factor

In the flocks colonised at slaughter, no statistically significant difference was observed in the *Campylobacter* counts on carcasses when birds tested positive halfway through the production cycle compared with flocks which tested negative halfway (Two-sample Wilcoxon rank-sum (Mann-Whitney) test z = -0.163; p=0.870).

4.3.5. Campylobacter detection and enumeration on breast meat: superficial and internal contamination of breast meat fillets

One hundred breast meat fillets were examined for superficial contamination during the whole study (25 were collected during the first production cycle, 44 during the second, and 31 during the third). Ninety-six fillets were investigated for deep contamination (25, 36 and 35 for each cycle respectively).

Regarding the internal contamination of the muscle, the microbiological analysis of the rinse showed 3 failures in reaching a complete sterilisation of the surface of the breast meat fillets and 3 positive samples for deep contamination were excluded from the analysis for this reason. Because the verification of the sterilisation procedures were put in place just after the first production cycle, data collected for internal contamination during the first crop were excluded from the analysis, as the prevalence of 15% after 48 hours of incubation in modified Exeter broth could potentially include contamination from the surface of the breast fillets.

By direct plating, 8% of breast fillets analysed were positive for superficial contamination. Significantly higher *Campylobacter* detection on breast meat surfaces was reported after including any enrichment step ($p<0.001$). Comparing the different broths and the different incubation times, a higher *Campylobacter* prevalence was observed after enrichment in modified Exeter broth for 48 hours, with a prevalence of 46% reported. However, no significant difference was observed among the different

broths and the different incubation times (Figure 4.8). Average mean count on the surface of the breast meat was 0.157 log cfu/g (95% CI 0.0418 - 0.271).

Compared to the contamination on the breast surface a lower *Campylobacter* contamination in the internal muscle was detected. No positive samples were reported for deep contamination by direct plating, and a 4% prevalence of internally contaminated fillets was observed after enrichment in Bolton and modified Exeter broths after 48 hours (Figure 4.8).

Among the 8 *Campylobacter* positive breast meat samples by direct plating for superficial contamination, only one sample belonged to a *Campylobacter* negative flock. Positive samples for internal contamination were observed only from colonised flocks.

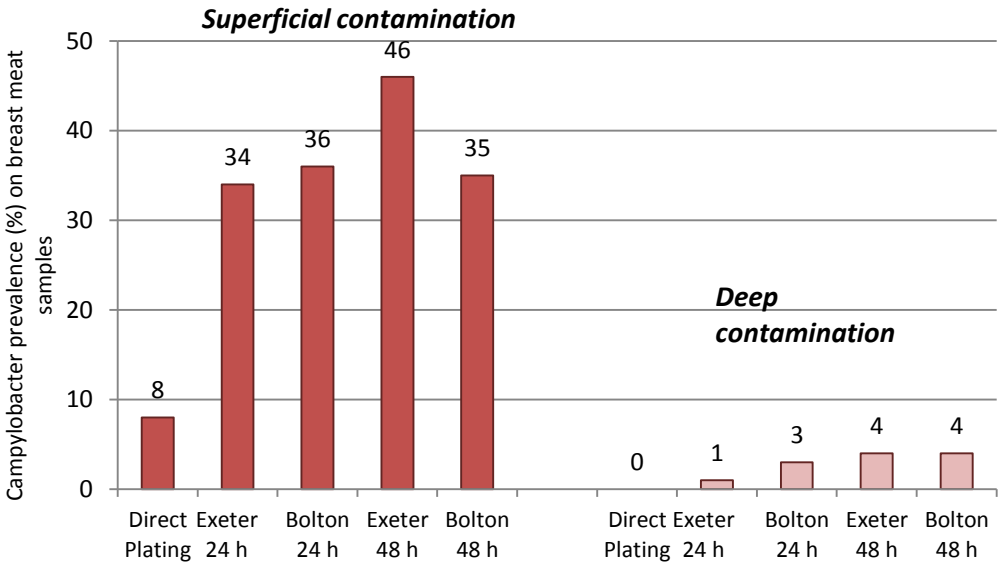

Figure 4.8: Percentage of Campylobacter positive breast meat samples (superficial and deep contamination) by different detection method

Overall, 134 *Campylobacter*-like isolates were identified by multiplex PCR as described by Klena et al. based on differences in the *lpxA* gene, on breast meat samples (Klena, Parker et al. 2004). A more detailed description of the typing methods used is included in Chapter 5. Among the whole population, 105 isolates were *C. jejuni* (78%) and 29 were *C. coli* (22%). Other authors have described similar levels for *C. jejuni* and *C. coli* distribution on breast meat samples (Atanassova, Reich et al. 2007; Luber and Bartelt 2007; Rahimi, Kazemeini et al. 2010; Bardoňa, Kolářb et al. 2011). No significant difference in the *Campylobacter* species distribution was observed between isolates collected from the surface of the breast meat (76% *C. jejuni*, 24% *C. coli*) and isolates collected from the internal muscle (92% *C. jejuni*, 8% *C. coli*) (Table 4.6).

Breast meat isolates were collected from all culture methods to investigate if different detection methods and enrichment steps were also affecting the *Campylobacter* species distribution. Contrary to what was reported by William et al. in 2012, the distribution of *Campylobacter* species appeared not to be influenced by any different detection and enrichment method (Williams, Sait et al. 2012) (Table 4.6).

	Superficial v. Deep contamination		**Direct plating v. Enrichment**		**By Broth**		**Exeter Broth**		**Bolton Broth**	
	Sup. Cont	Deep Cont	Direct Plating	Enrich.	Exeter	Bolton	Exeter 24 h	Exeter 48 h	Bolton 24 h	Bolton 48 h
C. jejuni	76%	92%	86%	77%	80%	70%	77%	83%	76%	65%
C. coli	24%	8%	14%	23%	20%	30%	23%	17%	24%	35%

Table 4.6: C. jejuni and C. coli distribution by sample part, detection method and enrichment step

4.4. DISCUSSION

In both production systems, apart from day one, *Campylobacter* was isolated at all stages during the rearing period. Both production systems were able to rear *Campylobacter*-negative flocks until the first depopulation event. However, all samples from Standard flocks collected at slaughter after thinning were *Campylobacter* positive.

As expected from previous studies, thinning, which is the partial depopulation of the flock that allows for the weight of the remaining birds to be increased before slaughter, appeared to be a significant risk factor for *Campylobacter* colonisation for the residual birds (Hald, Rattenborg et al. 2001; Adkin, Hartnett et al. 2006; Vandeplas, Dubois-Dauphin et al. 2010; Patriarchi, Fox et al. 2011). One explanation for this is that it is very difficult for the thinning crews to maintain reasonable levels of bio-security and hygiene during the operations (Ramabu, Boxall et al. 2004). A particular area of concern is the lack of cleanliness and disinfection of the crates used for the transportation of the animals. Different studies reported that crates used for the transport of birds were returned to the farms still contaminated with *Campylobacter*, despite the fact that they were washed and disinfected in the processing plants as per EU Regulation 853/2004 (Slader, Domingue et al. 2002; Herman, Heyndrickx et al. 2003; Hansson, Ederoth et al. 2005; Ellerbroek, Lienau et al. 2010). Therefore, contaminated crates entering into the broiler house increase the risk of *Campylobacter* infection for the remaining flock which will be slaughtered around 7-14 days later. In addition the stress that birds undergo during thinning due to feed withdrawal (Mench 1991) and the stress suffered by the animals due to the ingress of catchers and their

equipment, might support the colonisation and the spread of the bacteria through the remaining animals, as it is well known that the stress decreases the animals' ability to cope effectively with bacterial infection (Leitner and Heller 1992; Rosales 1994). Furthermore, such stress is expected to increase the level of neurotransmitters, such as noradrenalin, which are believed to stimulate the growth and motility of *C. jejuni* enhancing further *Campylobacter* colonisation in the remaining animals (Lyte and Ernst 1992; Humphrey 2006; Cogan, Thomas et al. 2007; Rostagno 2009).

Consequently in light of the above findings, the poultry industry needs to pay more attention to the practice of thinning. Countries such as Iceland and Finland, with an effective *Campylobacter* control plan, do not carry out partial depopulation of the flocks (Perko-Mäkelä, Hakkinen et al. 2002; EFSA 2008). However, the EU Directive EC 2007/43, which regulates the stocking density in broilers farms, makes it more difficult for the European poultry industry to eliminate this practice during the rearing period. In fact, to increase the profitability, the majority of UK farms are initially filled with around 30% more birds than is allowed by the above regulation and the extra birds are then removed before the final stocking density exceeds the welfare limits (approximately 1-2 weeks before the final depopulation).

If in the UK poultry industry, thinning cannot be avoided for economic reasons, the need for better hygiene control of the equipment and of the personnel involved in the operations becomes crucial. In particular, extra care must be taken during the cleaning and disinfection of the plastic crates at the slaughterhouses as due, to their design, crates have recesses that can trap organic material and bacteria such as *Campylobacter*. One area identified for improvement, would be to make the crate

washing machines work more efficiently. Therefore the temperature of the water, recycling water system, concentration of chemicals and length of the washing cycle are all factors that need to be considered when designing these machines to achieve an effective removal of the existing contamination. Berrang et al. in 2004 reported that storing the crates for 48 hours between uses resulted in lower numbers of bacteria on crates (Berrang, Northcutt et al. 2004). However, due to the cost of the crates and space needed, longer storage between uses is not practical. Moreover, the use of disposable crate linings needs to be considered to prevent the accumulation and to facilitate the washing operations.

To make matters worse, together with the problem highlighted above, it was noticed during the visits of all three company farms that after the first depopulation event farm staff and management appeared less inclined to implement bio-security practices. The management's perception was that a break in the bio-security barriers had already been caused by the catching team, therefore maintaining high levels of bio-security procedures was seen as a pointless exercise for the rest of the rearing period.

With the aim to avoid further stress to the animals during the rearing period (Nicol and Scott 1990), partial depopulation is strongly discouraged by the Freedom Food Scheme and from the 1st January 2016 the practice of thinning will not be permitted in flocks raised under this welfare scheme (RSPCA 2011). However, according to the poultry producer internal policy, this practice was already not allowed among the Freedom Food flocks investigated in this study. This eliminated a significant risk factor for *Campylobacter* colonisation in this production system. However, the public health benefits of not carrying out partial depopulation of the flock in the Freedom Food

farms cannot be observed if in the same farm yard Standard birds are reared at the same time and no strict hygiene barriers are in place to separate completely the two production systems. In Farms 4 and 5, for example, the placement of Standard and Freedom Food chicks was carried out on the same day. In the Standard flocks, the first thinning was carried between 37 to 41 days, and the final depopulation of the house was carried out 8 to 13 days later together with the depopulation of the whole Freedom Food flocks. Bearing in mind that the effect of other farm parameters has not been controlled during the analyse, from our data it emerged that in the same way the thinned Standard flocks were always colonised at slaughter, the potential negative effects of the ingress of the catching team in the farm were also observed among the Freedom Food flocks that appeared always negative at half cycle, but they had a 100% of flock colonisation prevalence at slaughter.

Two other Freedom Food farms (Farms 2 and 7), kept animals of both production systems of different ages at the same time. However, in this case the management appeared to be completely aware of the risk that could be incurred by staff movements between flocks of different ages and different production systems. The use of a shower, for example, was observed when a member of staff attending an older flock was requested for chick placement. In these farms, all the flocks were negative at slaughter. A Standard flock belonging to Farm 2 was also investigated at slaughter during the cross sectional study (Chapter 2) and it was found to be negative as well.

These findings suggest that in some farms, regardless of the presence of important risk factors for *Campylobacter* colonisation such as having broilers of different ages on site and the 'all in all out' policy not in place (Berndtson, Danielsson-Tham et al. 1996), it

was possible to deliver *Campylobacter*-free chickens at slaughter. Despite the fact that it is widely accepted that good bio-security measures are essential to keep flocks *Campylobacter*-free (Humphrey, Henley et al. 1993; Berndtson, Emanuelsonb et al. 1996; Evans and Sayers 2000; Hald, Wedderkopp et al. 2000; Advisory Committee on the Microbiological Safety of Food 2005; Allen and Newell 2005; Newell, Elvers et al. 2011) there were no other obvious reasons as to why some farms were more successful in producing *Campylobacter* negative flocks. A deeper investigation of the farm practices and management systems could better expose the grounds that are contributing to keep the flock *Campylobacter*-free and the findings could be exploited for disease control and to design more effective farm-based interventions.

In contrast with the results noted within the Freedom Food farms (where we have two farms which tested positive at slaughter for all the three production cycles and two farms which always tested negative), Standard farms showed very inconsistent results regarding flock colonisation status during the different production cycles. There were no logical explanations why farms were able to produce *Campylobacter*-free or colonised flocks during the different production cycles. On one occasion when the flock tested positive, the farmer blamed the flock infection on the fact that staff from a nearby farm helped whilst he was on holiday. This could be a plausible explanation for the flock colonisation. However, in other cases it was impossible to identify the reasons why in the same farm, run by the same management in the same conditions with no special issues reported, there were variations of outcome in flock colonisation. As suggested previously, a more comprehensive research of the farms' daily procedures must be undertaken, to understand where and why the failures occur or if the entry of *Campylobacter* into a poultry flock could be potentially linked to a chance

event. The identification of the sources of flock colonisation would enable to target bio-security measures towards the areas posing the greatest risk.

In commercial broiler production systems, together with environmental and management conditions, most of the welfare issues are direct consequences of genetic selection for a faster and more efficient production of chicken meat (Rauwa, Kanisb et al. 1998). In this study a large prevalence of animal welfare issues such as a higher house mortality, pododermatitis, hock burns and rejections at slaughter were observed among the faster growing breed reared under the Standard production system (Allain, Mirabito et al. 2009). In addition, in faster growing breed, an association between flock colonisation and welfare issues, such as pododermatitis and in particular hock lesions was observed. However, the association between *Campylobacter* infection, breed and welfare issues was analysed only at univariable level and other farm parameters need to be considered to explore the link between *Campylobacter*, welfare issues and bird type.

In agreement with other longitudinal studies, the fact that colonised flocks were followed by negative ones showed that the down time between the end of one production cycle and the next one, together with accurate cleaning and disinfection operations, were successful in achieving the elimination of *Campylobacter* from inside the broiler house hence removing the risk of carry-over of infection in consecutive flocks (Evans and Sayers 2000).

The significant increase in the *Campylobacter* counts reported at slaughter compared with the counts reported before the start of the catching operations, suggests that catching and transport have an influence on the level of flock colonisation at slaughter.

It has been observed in several studies that broiler chickens are stressed by the catching and the transport operations (Mitchella and Kettlewella 1994; Stern, Clavero et al. 1995). In our study, the policy of the poultry producer recommended that, before transport, feed was withdrawn from the flocks for six hours, in order to reduce the content of the gastrointestinal tract and consequently the volume of faeces excreted during transportation or spilled during the slaughter process (Wabeck 1972). It was highlighted in several studies that the stress caused by this fasting time, together with the ingress of the catching team with their bulky equipment inside the poultry house, the loading/crowding of the animals inside the plastic crates, the changes in temperature and finally the transport, increases the corticosteroid levels and peristaltic movement in the gut of the chickens. In addition, the stress is also responsible for a decrease in the strength of the wall of the intestinal tract. All the above factors are resulting in an increase of *Campylobacter* shedding at slaughter (Stern, Clavero et al. 1995; Whyte, Collins et al. 2001).

Since the Freedom Food flocks showed mostly a significant increase in the *Campylobacter* count in caecal samples after the transport, it would seem that the stress experienced during transport by the animals belonging to this scheme has a stronger impact on their wellbeing. This could be explained by the fact that transport is potentially the first major stress encountered by these animals in their life. This is in contrast to the Standard birds, which have been already exposed to more stressful living conditions during the whole rearing period.

With the current practices, it would be difficult to minimise the stress level of livestock during transport as the farms are often located far away from the slaughterhouses. In

this study we have still observed the negative effects of transport on the rise of *Campylobacter* counts at caeca level, despite the fact that all the farms were located within a 40 miles radius of the slaughterhouse. With the above data, no further comparison could be done on the detrimental effects of long distances against shorter ones.

At present, there is no time limit for the transport of live birds, and the only regulation in place is the EU Directive 1/2005 on the protection of animals during transport and related operations, which sets to a maximum of 12 hours for poultry feeding and watering intervals. It would be challenging to enforce measures compatible with business purposes that could reduce the stress during the transport. For example, over the last few years, the number of slaughterhouses in the UK has decreased due to the economic climate, tough competition and lack of profitability. Consequently only a few large slaughterhouses remain in the country. As a result slaughterhouses and processing facilities have become more centralised with increased productivity to remain competitive in the market. This new situation is forcing the birds to be transported longer to reach the processing plant.

In addition to the above welfare problem and its repercussion on *Campylobacter* level, these large centralised slaughterhouses could interfere further with the dynamic of the *Campylobacter* in the slaughterhouse environment. These processing plants have had to introduce longer and continuous production shifts which reduce the 'down time' normally dedicated to cleaning purposes, increasing the risk of accumulation of pathogens during the extended production shifts. Furthermore, this production optimisation based on profitability could lead to the downgrading of the standard

hygiene practices during the processing (International Consultative Group on Food Irradiation 2009). For example lack of regular breaks cleaning during the operations, lack of adequate line maintenance, inadequate staff turnover during the production day, are all factors that could increase the risk of *Campylobacter* contamination of the end products.

With the exception of one Standard flock (Long ST3.1) where we noted a slight increase in the *Campylobacter* level after the first depopulation, in the rest of the Standard flocks we observed the trend on *Campylobacter* level that we expected to see in the *Campylobacter* flock colonisation during the rearing period (Herman, Heyndrickx et al. 2003). *Campylobacter* was less detected in younger birds probably because of the presence of *Campylobacter*-specific maternal antibodies that are widely present in young chickens (Sahin, Naidan et al. 2003; Vandeplas, Dubois et al. 2009). There was, then, an increase in the number of *Campylobacter* after the catching and the transport and a final slight decrease in the number of these bacteria in older animals after the first depopulation. It has been reported in other studies that the development of an adaptive immunity and changes in the intestinal microflora could be involved in the decrease of the number of bacteria and in the number of colonised birds observed in older flocks (Achen, Morishita et al. 1998; Vandeplas, Dubois et al. 2009).

As the majority of the flocks become colonised at the end of the rearing period, if the slaughtering schedule of the animals was to be organised according to the flock's *Campylobacter* status, this would imply that the test to identify the flock status has to be done as close as possible to the slaughtering date. Logistic slaughter, according to

the *Campylobacter* status of the flock, is routinely used in some European countries such as Norway, Iceland, and Denmark (Stern, Clavero et al. 1995; Barrios, Reiersen et al. 2006; EFSA 2011). However, this practice would not necessarily have a big impact in terms of preventing cross-contamination during the processing, as we observed that the numbers of bacteria tended to be low on any cross-contaminated carcasses belonging to negative flocks (Nauta, van der Fels-Klerx et al. 2005; Johannessen, Johnsen et al. 2007; Elvers, Morris et al. 2011). The scheduled slaughter could be effective (Rasschaert, Houf et al. 2006) in particular when combined with the diversion of the products from *Campylobacter* positive flocks towards further processing such as freezing or cooking which could eliminate or reduce the number of the pathogens on the final products (Stern, Clavero et al. 1995; Stern, Hiett et al. 2003; Barrios, Reiersen et al. 2006; Georgsson, Thornorkelsson et al. 2006; Wagenaar, Mevius et al. 2006; Meldrum and Wilson 2007; FSA 2009).

The way the poultry industry is currently organised in the UK, with the slaughtering schedule of flocks planned up to one year in advance, together with the higher *Campylobacter* prevalence reported in the UK flocks, make the proposal to process flocks according to *Campylobacter* status not practical. Because of the various contributors involved in the poultry food chain management, that includes parent stocks, rearing farms, hatcheries, growing farms, catching teams, live birds transport vehicles management etc., it would be impractical to change the slaughtering schedule at the last minute according to the *Campylobacter* status, in particular as currently there is no statutory requirement to enforce it. According to the UK National Control Programme (NCP) for *Salmonella* in broiler flocks (DEFRA 2009), that sets out the statutory requirements for the monitoring and control of *Salmonella* contained in the

EU Zoonoses Regulations 2160/2003 and 646/2007, logistic slaughter is used when the flock is known to be *Salmonella* positive. In such case, the flock is slaughtered at the end of the day or if for logistic reasons the *Salmonella* positive flock cannot be moved at the end of the day, the processing of the positive flock is followed by a deep cleaning and disinfection of the processing line. Furthermore, if the flock is positive for *Salmonella* Enteritis or *Salmonella* Typhimurium a complete change of the water inside the scald tanks is also required. The low *Salmonella* prevalence among the UK poultry flocks (Cogan and Humphrey 2003; DEFRA 2011; O'Brien 2013), together with the requirement for the 'on-farm testing' of each flock during the three week period before slaughter, have facilitated the enforcement of this rule in the UK.

As observed in other studies, counts on caecal samples at slaughter appeared to be related to flock colonisation halfway through the flock production cycle (Hansson, Pudas et al. 2010). Among the *Campylobacter* positive flock at slaughter, a significantly higher count on caecal samples was observed when flocks were found positive at half cycle. This appeared in both production systems. This result showed that a delay in the flock colonisation can result in lower levels of *Campylobacter* entering into the processing plant.

Fresh retail chicken breast fillets were examined for detection and quantification of *Campylobacter* in order to establish the contamination on the fillet surface and within the deep pectoral muscle. The aim of using different *Campylobacter* detection methods was to determine which techniques, broths and incubation times were more effective for *Campylobacter* detection in breast meat samples. Contrary to the findings reported from Habib et al. in 2008 and in the national survey undertaken by the FSA

between May 2007 and September 2008, our study shows that *Campylobacter* detection in breast meat samples appeared most successful after an enrichment step (Habib, Sampers et al. 2008; FSA 2009). All samples which tested positive by direct plating were in fact positive also after enrichment. On the other hand, samples that were negative by direct plating were found to be positive after enrichment. As reported by other authors, recovery of *Campylobacter* after selective enrichment broths appears to be higher compared to direct plating, when low numbers, injured and/or stressed bacteria and also low background flora are expected to be reported (Beuchat 1986; Corry, Post et al. 1995; Mason, Humphrey et al. 1999; Abulreesh, Paget et al. 2005). Evaluating the different enrichment steps, incubation in modified Exeter broth for 48 hours appeared to maximize *Campylobacter* recovery from the breast meat fillets. However, comparing the different broths and the various incubation times, no significant differences were observed. As well enrichment broths and incubation times appeared not to have any effect on *Campylobacter* species distribution (Williams, Sait et al. 2012). The lower number of positive samples detected by direct plating may also be due to the fact that the majority of the samples had *Campylobacter* counts below our detection limits of 100 cfu/g, highlighting an inability of the enumeration method used to count low bacteria numbers. However, since this pathogen has a low infectious dose for humans (Robinson 1981; Black, Levine et al. 1988; Advisory Committee on the Microbiological Safety of Food 2005), corrective actions need to be taken to improve the safety of the final product independently of the bacterial count observed.

Our results showed that 46% of breast meat fillets ready to reach the supermarkets were contaminated with *Campylobacter* on the surface. This data was lower compared

to the figures reported in the last FSA 2009 survey, which highlighted a 65.2% *Campylobacter* prevalence in the UK poultry meat. At that time such high percentage of contaminated meat on the market prompted the FSA to rank *Campylobacter* as the most important organism to fight to reduce food-borne diseases in the UK. Consequently the FSA since 2010 has dedicated a large amount of their financial resources (£5.1 million) to the research on *Campylobacter* (FSA 2013).

Quantitative results, using only positive samples, showed a lower average mean count on the surface of the breast meat (2.24 log cfu/g) compared to the average count reported in the neck skin samples (3.23 log cfu/g). Since chicken skin also reaches the final consumer, it is important to identify which poultry products available on the supermarket shelves are posing a greater risk to the consumers. It is well known that during processing it is the skin together with the internal cavity of the broiler carcasses that is mostly exposed to faecal material and other potential sources of the bacteria such as contaminated equipment and dirty protective clothing worn by staff (Berndtson, Tivemo et al. 1992; Luber, Brynestad et al. 2006; Davis and Conner 2007; Sampers, Habib et al. 2008). In different surveys higher *Campylobacter* prevalence and *Campylobacter* counts were observed in skin-on products (Pointon, Sexton et al. 2008; FSA 2009). Consequently, to reduce effectively the risk of exposure to *Campylobacter* for the final consumer, it would be advisable to sell chicken products without skin. However, this remedial action would be very difficult to enforce due to the high consumer demand for the whole chicken and for a large range of various skin-on chicken products.

Nevertheless, breast meat contamination is linked to the flock colonisation status and the majority of contaminated breast fillets belonged to *Campylobacter* colonised

flocks, different routes are leading to the contamination of different parts of the breast meat. The surface of the breast fillet becomes contaminated due to cross-contamination during the processing. Whereas the deep muscle contamination is potentially due to the spread of the bacteria from the intestine through the blood (Sanyal, Islam et al. 1983). Richardson et al. in 2011, detecting *Campylobacter* in the circulating blood of commercial broilers brought to light the blood as a vehicle of dissemination for *Campylobacter* into organs (Richardson, Cox et al. 2011).

Overall in our study 4.26% of the fillets were contaminated with *Campylobacter* within the muscle tissue. However, as explained above, the way by which the deep tissue comes in contact with this pathogen, leads us to believe that only breast fillets belonging to colonised flocks can harbour *Campylobacter* in the deep tissue. For this reason, only breast meat samples from positive flocks were taken into account to estimate the bacteria spreading rate into the deep muscle tissue, and a 7.69% of internally contaminated breast fillets were reported.

Despite several studies showing the presence of *Campylobacter* from organs other than the intestinal tract (Cox, Hofacre et al. 2005; Cox, Richardson et al. 2006; Knudsen, Bang et al. 2006; Luber and Bartelt 2007; Jennings, Sait et al. 2011), further investigations are needed to understand which factors are involved in the spread of the bacteria from the chicken intestine to other parts that are destined for human consumption. There is increasing evidence that the bacteria can leave the chicken intestinal tract by breaking down the tight junctions of the epithelial cells (Knudsen, Bang et al. 2006; Van Deun, Haesebrouck et al. 2007). The role of stress, poor animal welfare and endemic diseases has been linked to this extra-intestinal spread of *Campylobacter*. The health and welfare of the chickens have a significant impact on

the ability of the immune system to control infections (Dohmsa and Metzb 1991; Rosales 1994; Mashaly, Hendricks et al. 2004). Furthermore, the hormone norepinephrine which is released into the lumen of the intestine during stress, has been shown to increase bacterial growth in vitro (Lyte and Ernst 1992), increasing also its virulence and its motility that allows bacteria penetration and colonisation of the epithelium (Methner, Rabsch et al. 2008). This suggests that the presence of this stress-related hormone could favour the bacterial ability to cross over the intestinal layer via the circulating blood into other organ tissues including the chicken meat (Humphrey 2006; Cogan, Thomas et al. 2007; Rostagno 2009).

There is evidence that fast growing breeds have less healthy gut compared to slow growing breeds. This could contribute to a more invasive *Campylobacter* infection in these fast growing birds. However in one recent study the impact of the breed on the effect of the extra-intestinal spreading was investigated and no differences between the breeds were reported. This study showed that breed alone is not a factor for extra-intestinal spread of *Campylobacter*, and that other environmental and management factors may contribute to the spread of the bacteria (Williams, Sait et al. 2012). In our study the seven internally contaminated breast fillets were collected from 2 different slower growing breed flocks (FB2.2 and FB2.3) belonging to the same Freedom Food farm (Farm 5). These two flocks at slaughter had low house mortality, rejections, pododermatitis and hock burns (flock FB2.2: 1.05 %, 0.13%, 0%, 0% and flock FB2.3: 1.51%, 0.26%, 1%, 0%). Interestingly, among all the flocks investigated, these two flocks reported the highest increase in the *Campylobacter* level after transport. It can be assumed that acute stress inflicted on the birds during catching/transport has led bacteria to adapt into a more pathogen-like behaviour, thereby enhancing the bacteria

growth in the intestinal tract and its ability to spread into other edible parts. Once again, the Freedom Food flocks seem to be more susceptible to acute stress rather than the Standard birds. To understand better the changes in the bacterial behaviour and the host immune response as reaction to acute and chronic stress factors, it would be crucial to identify which factors are responsible for the different outcomes in the chicken infection during the poultry production.

From a food safety point of view, deep *Campylobacter* contamination of parts intended for human consumption identifies a further threat to public health, emphasizing two different pathways for consumer exposure to this pathogen. Bacteria present on the surface of the fillet can be easily destroyed by cooking, and unhygienic kitchen practices leading to cross-contamination appear to be the major risk for the consumer when contamination is found on the meat surface, as the bacteria spread easily in the kitchen environment (Cogan, Slader et al. 2002; Kusumaningrum, Riboldi et al. 2003; Luber, Brynestad et al. 2006). On the other hand, bacteria present within the fillet muscles are posing a risk to the consumer mainly if the meat is undercooked. In conclusion, as we found a higher *Campylobacter* prevalence on the surface of the breast meat fillets compared to the contamination reported within the muscle, it would be insufficient to only focus people's attention on the risk of undercooking chicken meat (Hillers, Medeiros et al. 2003). Consumers must also be aware that improving kitchen hygiene during food preparation, for example using different equipment and working surfaces for cutting vegetables and raw meat, or having a clear separation of raw meat and ready-to-eat food, would be a more effective way in reducing the risk of human campylobacteriosis.

CHAPTER 5

GENETIC DIVERSITY OF CAMPYLOBACTER SPECIES IN DIFFERENT PRODUCTION SYSTEMS UTILISING MULTI-LOCUS SEQUENCE TYPING

5.1. INTRODUCTION

Campylobacter is a zoonotic bacterium and one of the main causes of human bacterial gastroenteritis in the world, with reported cases in the UK and EU rising each year (EFSA 2012; Tam, Rodrigues et al. 2012). The routes by which humans are exposed to *Campylobacter*, include ingestion of contaminated food and water or direct contact with infected animals or carcasses (FAO and WHO 2002; Neimann, Engberg et al. 2003).

Different approaches for source attribution of human campylobacteriosis, have been considered in the EU (EFSA 2008), including investigations of the occasional outbreaks, epidemiological studies of sporadic cases and microbial sub-typing (Kramer, Frost et al. 2000; Batz, Doyle et al. 2005). When used to determine transmission routes and sources of infection, there are a number of limitations to typing methods including the time required to perform them, inadequate discrimination, availability of reagents and poor reproducibility of methodology within and between laboratories (Colles, Jones et al. 2003). However, Multilocus Sequence Typing (MLST) was developed with the aim of achieving a high level of discrimination between isolates in several bacterial genera (Maiden, Bygraves et al. 1998) and has shown to be effective for *Campylobacter* source attribution (Dingle, Colles et al. 2001; Sails, Swaminathan et al. 2003; Wilson, Gabriel et al. 2008). Based on the sequences of different loci representing housekeeping genes under low neutral selective pressure, this typing method also has the advantage that data can be stored in a single database that can be interrogated electronically via the Internet to produce a powerful resource for global epidemiology (Dingle, Colles et al. 2001). MLST classifies each isolate as a sequence type (ST) according to the sequence

analysis of seven housekeeping genes, chosen as they encode for essential metabolic functions in a cell and are thus present in all bacteria (Maiden, Bygraves et al. 1998). They are also chosen as they have a slow turnover with regards to genetic drift. This genotyping method has already been developed for a range of bacteria and, when carried out on national-scale, it was a practical and efficient method for the quantification of the contribution of different sources to human *Campylobacter* infection in the UK. It showed clearly that the most significant risk factor associated with human *Campylobacter* infection was the presence of this organism on chicken meat (Wilson, Gabriel et al. 2008; Sheppard, Dallas et al. 2009).

Although it is well recognised worldwide that chicken meat is one of the major sources of human *Campylobacter* infection (Wilson, Gabriel et al. 2008; Mullner, Spencer et al. 2009), there is little information available on whether the contamination levels of *Campylobacter* on chicken meat -and therefore the risk of entry into the food chain- varies when birds are reared under different commercial production methods. MLST was used in this study in order to investigate the genetic diversity of *Campylobacter* isolates between different production systems. The main aim of this study was to identify a possible association of *Campylobacter* species and strains with particular broiler production method, and potentially attributing human infection to a definite broiler production system.

In addition, in order to investigate the diversity of *Campylobacter* strains on matching carcasses and breast meat, MLST was carried out on isolates collected in the final poultry meat products to determine if the same strain diversity reported in the caecal samples was also seen in the chicken products ready to reach the consumers.

Although the majority of *Campylobacter* contamination on carcasses seems to originate from the slaughtered flock itself, the dynamics of strain diversity on meat appears more complex. During processing, the carcasses are exposed to cross-contamination through air, water, slaughterhouse equipment and/or workers' hands. Different studies reported that carcasses sampled at the end of the processing line, had been contaminated with *Campylobacter* even when the bacteria were not isolated from the chickens on arrival at the abattoir (Berrang, Buhr et al. 2001; Newell, Shreeve et al. 2001; Johnsen, Kruse et al. 2007). Furthermore, it was shown that the subtypes of *Campylobacter* found on the carcasses from colonised birds were not always those which were most prevalent in the gut of the birds (Newell, Shreeve et al. 2001). Additionally during transport, birds are potentially exposed to *Campylobacter* through contaminated crates and the diversity of strains on the bird surface (on feathers and skin) could increase. Interestingly, not only are strains gained during processing, but strain diversity may also be reduced, presumably due to poor survival properties against environmental stressors (e.g. changes in temperature, oxygen and chlorine dioxide) of this pathogen along the food chain.

It appears evident that strains isolated in the final product may not reflect the original ones identified in the flock at the farm or even at slaughter. The second main aim of this work was to determine if there were some *Campylobacter* sequence types that have better survival/transmission through poultry processing as evidenced by their greater abundance and are posing a greater zoonotic risk to the final consumer.

5.2. MATERIAL AND METHODS

5.2.1. Sample collection

Campylobacter colonies were isolated from caecal, neck skin, breast meat and liver samples collected from different batches at slaughter at three different abattoirs in the UK, over a 2 year period from the 23rd August 2010 until the 07th July 2012.

Caecal samples were collected at the time of evisceration by manual traction at the junction with the intestine. Neck skin samples were collected at the end of the slaughtering line directly after the air chilling process and before any further processing. Liver samples were collected at the time of evisceration and breast meat samples were collected in the cutting plant from packed meat ready for despatching. All the samples were processed, individually, as previously described in Chapter 2 and Chapter 4, for qualitative/quantitative detection of *Campylobacter*.

5.2.2. Campylobacter isolates and preparation of DNA extraction

From all samples, presumptive *Campylobacter* colonies were identified directly on mCCDA plates by their typical appearance (grey, flat, irregular and spreading colonies), by microscopic examination following Gram Staining (Gram negative curved rods) and an inability to grow under aerobic conditions (HPA 2011).

From each *Campylobacter* positive sample, up to 5 presumptive colonies of different morphologies, were retained for later typing. Each single colony was sub-cultured onto

CAB and incubated under microaerobic conditions (MACS-VA 1000- microaerobic workstation: Nitrogen 80%, Hydrogen 3%, Oxygen 5%, Carbon Dioxide 12%) at 41.5°C for a further 48 hours. According to the work schedule, colonies were either put into Microbank vials (Pro-Lab Diagnostics) and stored at -70°C or immediately processed for genotyping. Studies in vitro have showed that the stability of *Campylobacter* strains is generally not affected by freezing, sub-culturing and storing (Wassenaar and Newell 2000) .

From both fresh isolates or ones recovered from frozen beads, chromosomal DNA was extracted by boiling a loop of the cultured colonies in 300µl of Chelex solution (Chelex-100,Bio-Rad) for 10 minutes (Walsh, Metzger et al. 1991). The solution was then centrifuged (Mikro 20, Hettich, Zentrifugen) at 13,000rpm for 3 minutes to form a pellet containing bacterial cell debris. 50µl of the supernatant containing the suspended DNA, was added to 450µl of fresh solution containing sterilised distilled water and stored at 4°C.

5.2.3. Isolates speciation

After the DNA extraction, isolates were confirmed and identified to species level *(C. jejuni or C. coli)* using a multiplex PCR as described by Klena et al. based on differences in the *lpxA* gene (Table 5.1) (Klena, Parker et al. 2004). Isolates negative for amplicons with the *lpx* PCR were analysed with a PCR assay specific for the genus *Campylobacter* on the basis of 16S rRNA gene sequences (16S rRNA), to confirm that they belong to the genus *Campylobacter* (Table 5.2) (Linton, Owen et al. 1996; Katzav, Isohanni et al. 2008). A further PCR test for identification of the genus *Arcobacter*

(Gonzalez, Garcia et al. 2000) was run on all isolates negative to the genus *Campylobacter* (Table 5.3) due to its close phylogenetic relationship to *Campylobacter* (Lehner, Tasara et al. 2005), its high prevalence on broiler carcasses and its potential as an emerging food borne pathogen (Corry and Atabay 2001; Humphrey, O'Brien et al. 2007; Son, Englen et al. 2007). All primers used in this work were obtained from Eurofins MWG Operon (Germany) and all PCR constituents were supplied by Thermo Scientific, Surrey.

Primer	Sequence 5' – 3'
lpxAC. Coli	AGACAAATAAGAGAGAATCAG
lpxAC. Jejuni	ACAACTTGGTGACGATGTTGTA
lpxARKK2m	CAATCATGDGCDATATGASAATAHGCCAT

Table 5.1: Primers used in the lpx gene PCR assay for Campylobacter isolate's speciation (Product size: C. coli 391 bp and C. jejuni 331 pb)

Primer	Sequence 5' – 3'
C412F	GGA TGA CAC TTT TCG GAG C
CampR2	GGC TTC ATG CTC TCG AGT T

Table 5.2: Primers used in the 16S rRNA PCR assay for identification of the genus Campylobacter (Product size 857-bp DNA)

Primer	Sequence 5' – 3'
Arc1	AGAACGGGTTATAGCTTGCTAT
Arc2	GATACAATACAGGCTAATCTCT3

Table 5.3: Primers used in the 16S rRNA PCR assay for identification of the genus Arcobacter

All PCR assays were performed in 25µl volumes, comprising 22µl 1.1X ReddyMix PCR Master Mix, 0.5µl of each primer (100 pmol/µl), and 2µl of DNA extraction. The following thermo cycling conditions were used for the PCR assays: one initial heating cycle of 94°C for 5 min followed by 30 amplification cycles consisting of 1 minute at 94°C (denaturation), 1 minute at 50°C (annealing) and 1 minute at 72°C (extension step), and a final cycle of 7 minutes at 72°C. The PCR products were then visualised by electrophoresis in a 1.5% agarose gel in in tris–acetate buffer (TAE) which was stained with ethidium bromide and exposed to UV light.

5.2.4. Multilocus Sequence Typing process

After species differentiation, around 150 isolates were selected ad hoc for sequencing and sequence type assignment. The MLST protocol comprised three main stages: PCR amplification of the 7 genes, PCR amplicon purification and DNA sequencing. The reaction method and primers used were initially based on the method described by Dingle et al. in 2000, which set specific primers for *C. jejuni* and *C. coli* (Dingle, Colles et al. 2001). For *C. jejuni*, the primers used were to detect portions of the genes encoding: aspartase A (*Asp A*), glutamine synthetase (*GlnA*), citrate synthase (*GltA*), serine hydroxymethyl transferase (*GlyA*), phosphoglucomutase (*pgm*), transketolase (*tkt*) and ATP synthase α subunit (*uncA*) (Table 5.4).

Locus	Primer	Sequence 5' – 3'	Amplicon size (Bp)
aspA	Forward	AGTACTAATGATGCTTATCC	899
	Reverse	ATTTCATCAATTTGTTCTTTGC	
glnA	Forward	TAGGAACTTGGCATCATATTACC	1262
	Reverse	TTGGACGAGCTTCTACTGGC	
gltA	Forward	GGGCTTGACTTCTACAGCTACTTG	1012
	Reverse	CCAAATAAAGTTGTCTTGGACGG	
glyA	Forward	GAGTTAGAGCGTCAATGTGAAGG	816
	Reverse	AAACCTCTGGCAGTAAGGGC	
Pgm	Forward	TACTAATAATATCTTAGTAGG	1150
	Reverse	CACAACATTTTTCATTTCTTTTTC	
Tkt	Forward	GCAAACTCAGGACACCCAGG	1102
	Reverse	AAAGCATTGTTAATGGCTGC	
uncA	Forward	ATGGACTTAAGAATATTATGGC	1120
	Reverse	GCTAAGCGGAGAATAAGGTGG	

Table 5.4: Primers used for C. jejuni MLST protocol from Dingle et al. 2001

To amplify each locus, from each isolate, seven amplification reactions were performed in 50µl volumes containing 36.75µl molecular grade water (Sigma-Aldrich Company Ltd., Gillingham, UK), 5.0µl Buffer IV, 3.0µl Magnesium chloride, 0.25µl Taq DNA polymerase, 1µl deoxynucleotide triphosphates, 1µl (100 pmol/µl) of the forward and reverse primers and 2µl of DNA extraction. The reactions were placed into thermal cycler (2720 Thermal Cycler Applied Biosystem) sets with an initial heating stage of 3 minutes at 94°C; followed by a second stage of 35 cycles consisting of 20 seconds at 94°C, 20 seconds at 50°C, and one minute at 72°C and a final step of 72°C for 5 minutes.

To verify whether the DNA amplification was successful, 5µl of PCR products was separated by gel electrophoresis using a 1.5% agarose gel in in tris–acetate buffer (TAE) stained with ethidium bromide, and visualised using ultra-violet light.

As the Dingle primers described above showed positive but inconsistent banding patterns between samples, a second MLST method described by Miller et al. 2005, was used to optimise the entire process (Miller, On et al. 2005). This second-generation of *Campylobacter* MLST scheme (Table 5.5) typed with the same primers *C. jejuni* and *C. coli*, making initial identification of species unnecessary.

Locus	Primer	Sequence 5' – 3'	Amplicon size (Bp)
aspA	Forward	GAGAGAAAAGCWGAAGAATTTAAAGAT	676
	Reverse	TTTTTTCATTWGCRSTAATACCATC	
glnA	Forward	TGATAGGMACTTGGCAYCATATYAC	751
	Reverse	ARRCTCATATGMACATGCATACCA	
gltA	Forward	GARTGGCTTGCKGAAAAYAARCTTT	706
	Reverse	TATAAACCCTATGYCCAAAGCCCAT	
glyA	Forward	ATTCAGGTTCTCAAGCTAATCAAGG	716
	Reverse	GCTAAATCYGCATCTTTKCCRCTAAA	
Pgm	Forward	CATTGCGTGTDGTTTTAGATGTVGC	716
	Reverse	AATTTTCHGTBCCAGAATAGCGAAA	
Tkt	Forward	GCAAAYTCAGGMCAYCCAGGTGC	730
	Reverse	TTTTAATHAVHTCTTCRCCCAAAGGT	
uncA	Forward	GWCAAGGDGTTATYTGTATWTATGGTTGC	700
	Reverse	TTTAADAVYTCAACCATTCTTTGTCC	

Tab 5.5: Primers used for C. jejuni and C. coli MLST protocol from Miller et. al.2005

To increase the speed of the whole process, and reduce the risk of contamination and pipetting errors, a 1.1X ReddyMix PCR Master Mix (Thermo Scientific) was used at a later stage to prepare the PCR amplification reactions with satisfactory results. Occasional failures to obtain complete banding patterns still occurred with the Miller protocol and PCR amplifications were repeated for the single failed allele until amplification was successful.

5.2.4.1. Polyethylene Glycol 'PEG' Precipitation

Following confirmation of successful PCR, the amplified products were purified by applying the PEG precipitation protocol. 60µl of 20% (w/v) polyethylene glycol, PEG 8000 (Sigma, Steinheim, Germany) containing 2.5M NaCl, was added to each amplified PCR product, and the sample mixtures were incubated at 37°C for 15 minutes, before being centrifugated at 2,750 rcf at 4°C for 60 minutes. In each sample, the supernatant was then removed and the pellet was washed twice with 150µl ice cold 70% ethanol, followed by a centrifugation step at 2,750 rcf for 10 minutes. Ethanol was then removed and samples were left to air dry on the bench for 10 minutes. Dried pellets were re-suspended in sterile milliQ water according to the intensity of PCR bands observed at UV light on the gel produced after the PCR stage. This varied from 5µl for weak bands to 35µl for stronger bands.

5.2.4.2. Sequencing

Purified PCR products were sent to Macrogen Inc. (South Korea) for sequencing using the same primer pairs used for the amplification of the products (Table 5.5). The sequences of MLST amplification products were then verified using Chromas Pro V1.7.3 (Technelysium Pty Ltd). Allele numbers and sequence types were assigned by investigating existing alleles in the MLST *C. jejuni* and *C. coli* database (http://pubmlst.org/campylobacter). Also, clonal complex relationships between genotypes were assigned using the pubMLST databank. Isolates were grouped in clonal complexes (CCs) on the basis of sharing four or more identical alleles (Dingle, Colles et al. 2001). To date there are a total of 6564 sequence type profiles in the database across the two species (Last updated: 02.05.2013). All of the new profiles identified in

this study were submitted directly to the PubMLST *C. jejuni/coli* database for ST assignment.

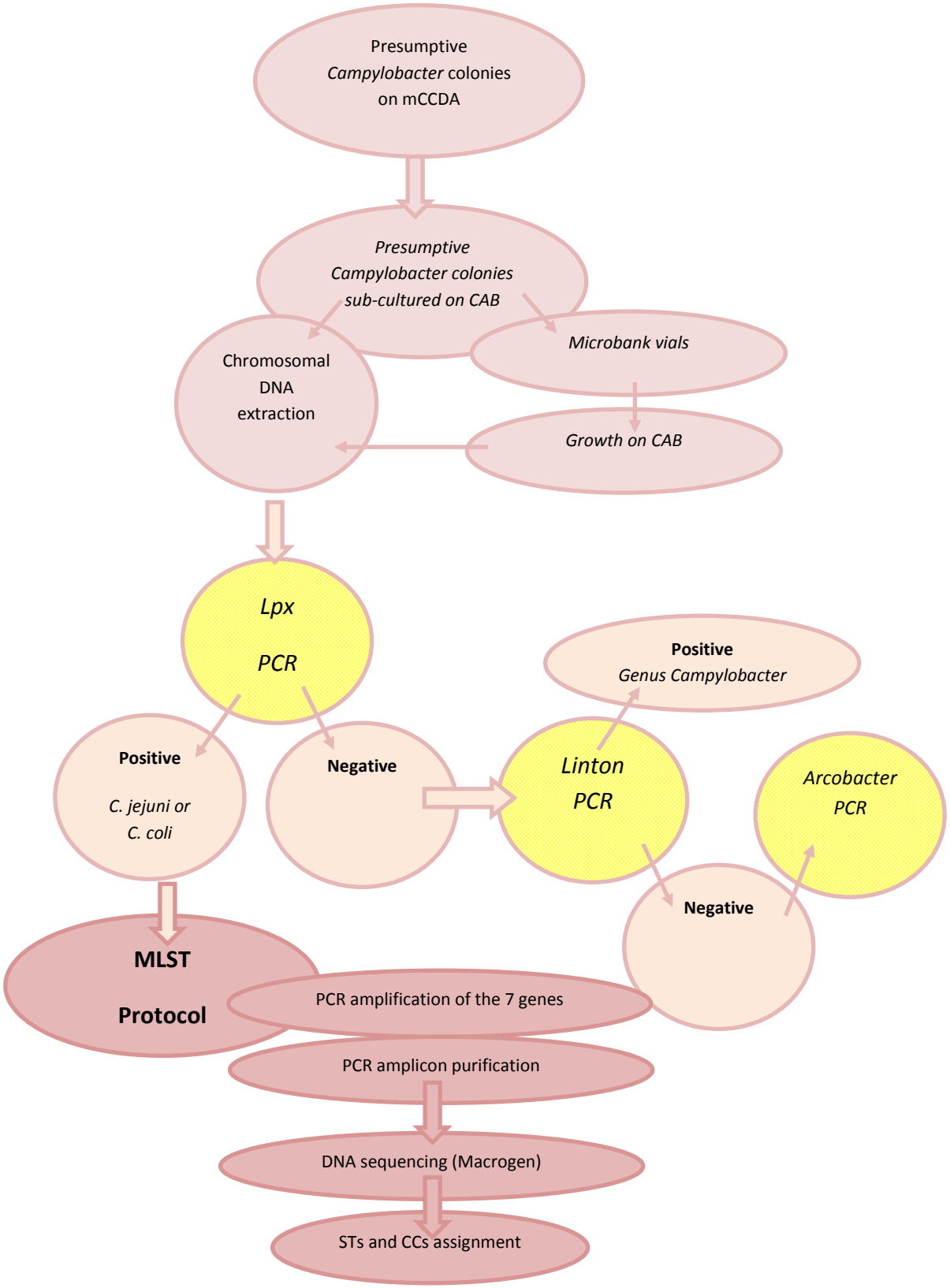

Figure 5.1: Flow chart showing PCR and sequencing steps

5.3. RESULTS

During the whole study, 1861 isolates were retained for *Campylobacter* species differentiation and further analysis. PCR assay for assignment of *Campylobacter* species was performed on 720 (38.7%) isolates. In total, 600 isolates gave positive results using the Klena multiplex PCR assay: 483 isolates (80.5%) were identified as *C. jejuni* and 117 (19.5%) were identified as *C. coli*. *C. jejuni* was the predominant *Campylobacter* species also when the analysis was carried out independently within each production system (Table 5.6) and by sample types (Table 5.7).

Production system	Number of isolates	*C. jejuni (%)*	*C. coli (%)*
Standard	241	184 (76%)	57 (24%)
Freedom Food Indoor	195	146 (75%)	49 (25%)
Improved Welfare	94	83 (88%)	11 (12%)
Freedom Food Free Range	70	70 (100%)	0 (0%)

Table 5.6: Proportion of C. jejuni and C. coli across the different production systems

Samples type	Number of isolates	*C. jejuni (%)*	*C. coli (%)*
Caeca	309	243 (79%)	66(21%)
Neck Skin	154	133 (86%)	21 (14%)
Breast Meat	134	105 (78%)	29 (22%)
Liver	3	2 (67%)	1 (33%)

Table 5.7: Proportion of C. jejuni and C. coli across the different sample types

Among the 120 isolates negative with the *lpx* multiplex PCR assay, 13 (10.8%) were still identified as *Campylobacter* species using the 16S rRNA PCR assay. Seventy one isolates (59.2%) were identified as *Arcobacter* spp and 31 (25.8%) gave negative results with both *Campylobacter* and *Arcobacter* PCR assays. Five isolates (4.2%) gave positive results to both *Campylobacter* and *Arcobacter* essays and considering a potential cross-reaction between Linton primers and some *Arbobacter* isolates, they were therefore likely to be *Arcobacter.*

Only isolates confirmed as *C. jejuni* or *C. coli* were further investigated and genotyped using MLST. As *C. jejuni* is the *Campylobacter* species predominantly found in broilers (Bull, Allen et al. 2006; Jorgensen, Ellis-Iversen et al. 2011) and the one found most often in human cases (Gillespie, O'Brien et al. 2002) the majority of isolates chosen for sequencing and ST assignment belonged to that species.

MLST was successfully completed on 141 isolates of which 112 (79.4%) were *C. jejuni* and 29 (20.6%) *C. coli* (Table 5.8). The majority of isolates analysed were retained from the caeca (n=77) and neck skin samples (n=49). However, 14 breast meat isolates and 1 liver isolate were also included in this study (Table 5.9). Regarding the distribution among the production systems, 49 isolates were isolated from Standard birds, 27 from the Freedom Food indoor system, 36 from the Improved Welfare system and 29 among the Freedom Food free range birds (Table 5.10). The distribution of isolates by season is shown instead in Table 5.11.

	Number of isolates	Number of existing sequence types identified	Number of new sequence types identified	Number of isolates assigned to a known clonal	Number of clonal complexes (CCs) identified
C. jejuni	112	32	3	107	13
C. coli	29	6	1	20	1

Table 5.8: Distribution of isolates by Campylobacter species

	Number of isolates	Number of existing sequence types identified	Number of new sequence types identified	Number of isolates assigned to a known clonal complex	Number of clonal complexes (CCs) identified
Caeca	77	26	2	68	12
Neck skin	49	21	2	43	9
Breast meat	14	11	0	14	8
Liver	1	1	0	1	1

Table 5.9: Distribution of Campylobacter isolates by sample type

	Number of isolates	Number of existing sequence types identified	Number of new sequence types identified	Number of isolates assigned to a known clonal	Number of clonal complexes (CCs) identified
Standard System	49	16	1	45	9
Freedom Food Indoor	28	10	1	21	8
Improved Welfare	35	14	2	32	11
Freedom Food Free Range	29	8	0	29	5

Table 5.10: Distribution of Campylobacter isolates by production system

	Number of isolates	Number of existing sequence types identified	Number of new sequence types identified	Number of isolates assigned to a known clonal complex	Number of clonal complexes (CCs) identified
Spring	23	11	0	22	6
Summer	17	8	0	15	6
Autumn	65	20	3	56	11
Winter	36	15	1	34	6

Table 5.11: Distribution of isolates by season

The 141 isolates were collected from 36 different batches of broilers and represented 26 different farms located in England, Wales and Scotland. Overall, within the 141 isolates, a total of 38 existing STs were detected (Table 5.8). A total of 4 novel STs were identified and submitted to the *C. jejuni*/coli MLST database and they were assigned to 4 new sequences types (ST6567, ST6558, ST6559 and ST6560) in order of detection. Complete MLST profiles obtained from the 141 isolates are shown in Appendix 7.

ST464 (12.1%), ST45 (8.5%), ST257 (7.1%), and ST828 (6.4%) were the most common STs reported in our data set (Table 5.12).The 42 STs (38 existing and the 4 novel STs) were grouped into 14 clonal complexes. 14 STs (5 *C. jejuni* and 9 *C. coli*) remain unassigned (UI) to any of the known clonal complexes (last database query, May 2013) (Table 5.12). The number of distinct STs in each clonal complex ranged from one (ST-443, ST-658, ST-661 and ST-1034 complexes) to a maximum of seven in ST-45 clonal complex (Table 5.12).

Within the *C. jejuni* population, ST-45 complex (consisting of 28 isolates) was the predominant clonal complex observed; followed by ST-464 complex (with 18 isolates), ST-257 complex (with 15 isolates) and ST-21 complex (12 isolates). In total, 65.2% (73/112) of *C. jejuni* isolates analysed by MLST were grouped into one of these four clonal complexes. Less genotypic diversity was observed among the *C. coli* population, although the number of isolates was lower. Among the *C. coli* population all STs assigned to a clonal complex were grouped into the ST-828 complex (20 isolates) (Table 5.12).

Species	Clonal complex	Numb (%) of isolates	Sequence type	Numb (%) of isolates
C. jejuni 112 isolates	ST-21	12 (8.5)	ST21 ST50 ST797 ST2135	8 (5.7) 1 (0.7) 1 (0.7) 2 (1.4)
	ST-22	1 (0.7)	ST22	1 (0.7)
	ST-45	28 (19.9)	ST25 ST45 ST137 ST233 ST1695 ST2197 ST2223 ST6558*	1 (0.7) 12 (8.5) 5 (3.5) 5 (3.5) 1 (0.7) 2 (1.4) 1 (0.7) 1 (0.7)
	ST-48	2 (1.4)	ST48 ST739	1 (0.7) 1 (0.7)
	ST-257	15 (10.6)	ST257 ST2030 ST3444 ST6033	10 (7.1) 3 (2.1) 1 (0.7) 1 (0.7)
	ST-353	9 (6.4)	ST5 ST400 ST2976 ST6560*	3 (21.3) 1 (0.7) 4 (2.8) 1 (0.7)
	ST-354	9 (6.4)	ST354 ST1489	8 (5.7) 1 (0.7)
	ST-443	4 (2.8)	ST51	4 (2.8)
	ST-464	18 (12.8)	ST464 ST5136	17 (12.1) 1 (0.7)
	ST-573	3 (2.1)	ST573 ST5148	2 (1.4) 1 (0.7)
	ST-658	1 (0.7)	ST1900	1 (0.7)
	ST-661	4 (2.8)	ST661	4 (2.8)
	ST-1034	1 (0.7)	ST1709	1 (0.7)
	Unassigned	6(4.3)	ST2211 ST6557* ST6559*	3 (2.1) 2 (1.4) 1 (0.7)
C. coli 29 isolates	ST-828	20 (14.2)	ST825 ST827 ST828 ST4422	6 (4.3) 3 (2.1) 9 (6.4) 2 (1.4)
	Unassigned	8 (5.7)	ST1761 ST4558	2 (1.4) 6 (4.3)

*Table 5.12: C. jejuni and C. coli sequence types and clonal complexes among 141 chicken isolates (*Novel sequence types identified in this study)*

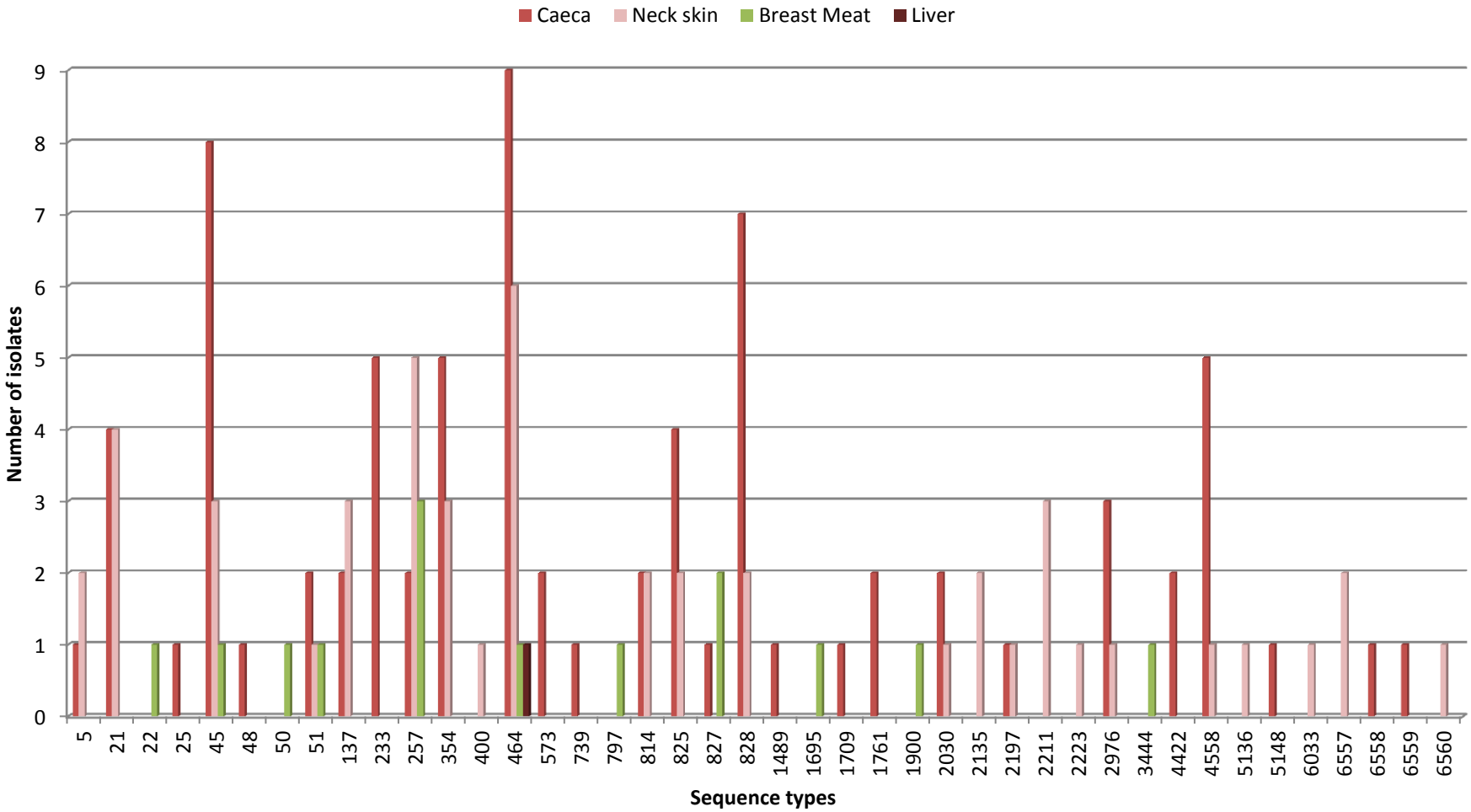

Figure 5.2: Distribution of C. jejuni and C. coli sequence types in different chicken sample types

More than 60% (26/42) of the sequence types identified in the data set, were observed only in a distinct sample type and only four sequence types (ST45, ST51, ST257 and ST464) were recovered from the 3 different types of sample (caecal, neck skin and breast meat) (Figure 5.2).

The distribution of *C. jejuni* and *C. coli* clonal complexes by sample type is given in Table 5.13, and represented graphically in Figure 5.3. Although all the clonal complexes reported in the neck skin samples were also observed in isolates collected from caecal contents, ST-22 and ST-658 complexes appeared only in isolates collected in the breast meat samples. In contrast, ST-48 complex, ST-573 complex and ST-1034 complexes were found only in the caeca. ST-257 complex that appeared to be the most common in breast meat samples (29%), and the second most common on neck skins (14%), was only the 5th most common among the twelve clonal complexes found in caeca (5%).

	Clonal complex														
	21	**22**	**45**	**48**	**257**	**353**	**354**	**443**	**464**	**573**	**658**	**661**	**828**	**1034**	**UI**
Caeca	4		18	2	4	4	6	2	9	3		2	14	1	8
Neck Skin	6		8		7	5	3	1	7			2	4		6
Breast Meat	2	1	2		4			1	1		1		2		
Liver									1						
All samples	12	1	28	2	15	9	9	4	18	3	1	4	20	1	14

Table 5.13: Distribution of C. jejuni and C. coli clonal complexes in different chicken samples (UI unassigned isolates)

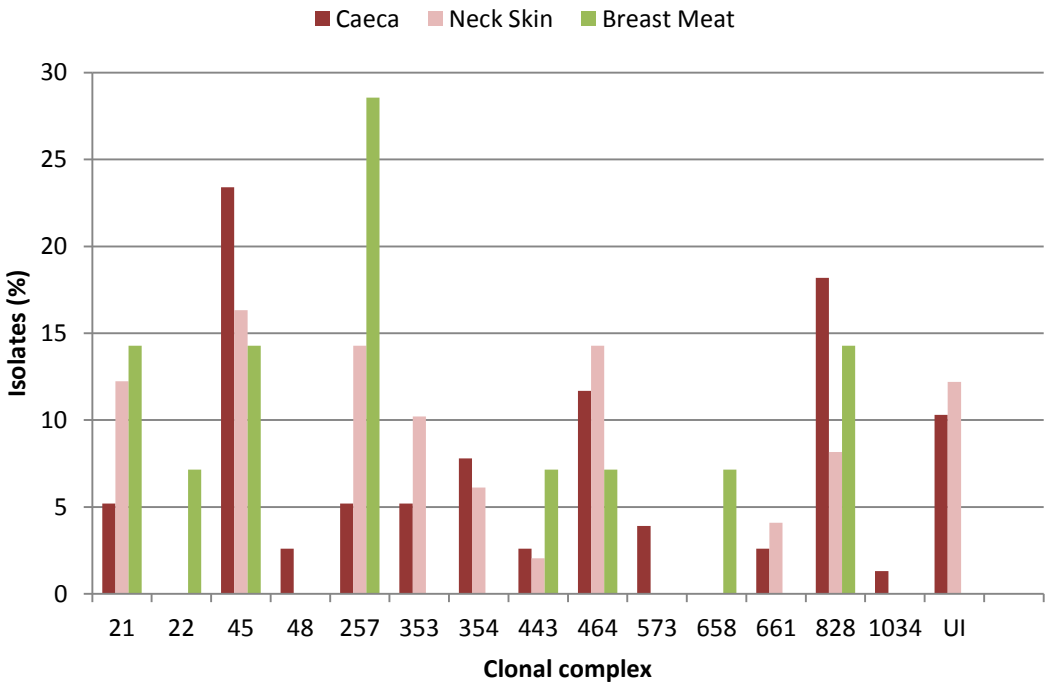

Figure 5.3: Distribution of C. jejuni and C. coli clonal complexes among caecal, neck skin and breast meat samples (UI unassigned isolates)

The genotypic diversity among the different samples (Table 5.14) based on the number of clonal complexes present, was quantified by the Simpsons index of diversity developed for the description of species diversity within an ecological habitat (Simpson 1494). It takes into account the number of CCs as well as the abundance of CCs. With this index, 0 represents infinite diversity and 1 no diversity. A large diversity was observed in the clonal complexes for each sample type (Table 5.14).

	Group	Number of isolates	Number of STs	Number of CC	Index of diversity
Production systems	Standard System	49	17	9	0.15
	Freedom Food Indoor	28	11	8	0.257
	Improved Welfare	35	16	11	0.13
	Freedom Food Free Range	29	8	5	0.425
Sample types	Caeca	77	28	12	0.119
	Neck skin	49	23	9	0.102
	Breast Meat	14	11	8	0.099
Seasons	Spring	23	11	6	0.245
	Summer	17	8	6	0.221
	Autumn	65	23	11	0.111
	Winter	36	16	6	0.289

Table 5.14: Genotypic diversity, based on the number of clonal complexes present, among commercial production systems, sample types and seasons
(for analysis of the production systems only caecal isolates were taken into consideration)

Seasonal variations in the distribution of different clonal complexes were observed. Clonal complexes ST-45 (50%) in the winter, ST-464 (47%) in the summer, ST-21 (22%) in the spring and ST-828 (22%) in autumn were the most prevalent clonal complexes reported across the year. ST-257 complex was the only one present in all four seasons within this dataset.

5.3.1. Distribution of genotypes among caecal isolates

Overall, within the 77 caecal isolates, ST464 (9 isolates), ST45 (8 isolates), ST233 and ST354 (5 isolates each) and ST21 (4 isolates) were the most common sequence types reported within the *C. jejuni* species. Within the *C. coli* species, the most dominant types were ST828 (7 isolates), ST4558 (5 isolates) and ST825 (with 4 isolates).

Within the *C. jejuni* population the largest clonal complex identified was ST-45 (consisting of 17 isolates), followed by ST-464 complexes (with 9 isolates), ST-354 complexes (with 6 isolates) and ST-21, ST-257 and ST-353 complexes (with 4 isolates each). In total 57% (44/77) of caeca isolates analysed by MLST were grouped into one of these six clonal complexes. Within the *C. coli* population all STs assigned to a clonal complex (14 isolates) were grouped into the 828 CC.

5.3.1.1. STs' and CCs' distribution in caecal samples in the different production systems

The distribution of STs in caecal samples within the different production systems is shown in Figure 5.4. The number of STs identified in each production system varied from 11 in the Standard system, 5 in the Freedom Food Indoor, 13 in the Improved Welfare System and 6 in the Freedom Free Range system. The most common sequence type found across each different production system was: ST464 (5/23) among the Standard system population, ST4558 (5/15) among the Freedom Food Indoor, ST233 (5/23) within the Improved Welfare system and ST45 (7/16) among the Freedom free range. No sequence type genotypes appeared to be common to all production systems (Figure 5.4).

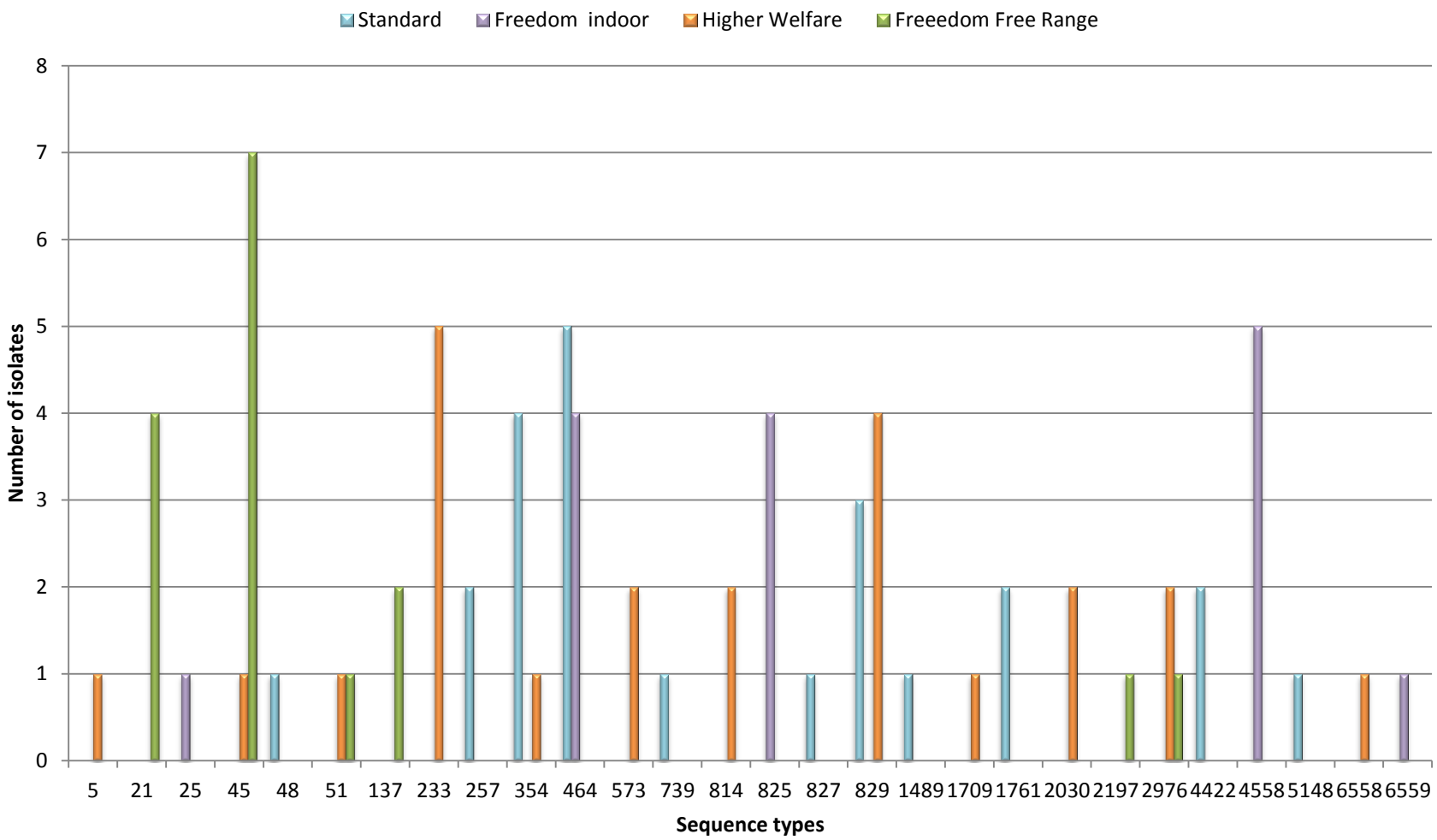

Figure 5.4: Distribution of C. jejuni and C. coli in caecal isolates within the different production systems

Except for the Freedom Food system, the rest of the systems exhibited at least one clonal complex exclusive to that production type: ST-48 complex was reported only within the Standard system, ST-661 and ST-1034 complexes only in isolates from the Improved Welfare system and ST-21 complex only among the Freedom Food free range population. The distribution of *C. jejuni* and *C. coli* clonal complexes in caecal samples by production types is given in Table 5.14. Among the different production systems, bigger diversity within the clonal complexes population was observed in the Improved Welfare system and Standard populations (Table 5.13).

	Clonal complex												
	21	**45**	**48**	**257**	**353**	**354**	**443**	**464**	**573**	**661**	**828**	**1034**	**UI**
Standard system			2	2		5		5	1		6		2
Freedom Food Indoor		1						4			3		6
Improved Welfare system		7		2	3	1	1		2	2	5	1	
Freedom Food Free Range	4	10			1		1						
Whole population	4	17	2	4	4	6	2	9	3	2	14	1	8

Table 5.14: Caecal isolates: frequency and distribution of clonal complex among production systems (UI unassigned isolates)

5.3.2. Neck Skin isolates

This molecular study of *Campylobacter* shows that strain diversity remains high throughout the food chain and the same strain diversity identified in the caecal samples appeared also in the neck skin samples (Table 5.13). Overall, within the 49 isolates, ST464 (6 isolates), ST257 (5 isolates) and ST21 (4 isolates) were the most common STs reported. The 23 STs were grouped into 9 CCs. Six isolates remained unassigned to a clonal complex (last database query, May 2013). The largest clonal complex reported among the neck skin isolates was ST-45 (consisting of 8 isolates), followed by ST-464 and ST-257 complexes (with 7 isolates each), and ST-21 complex (6 isolates) (Table 5.15). In total, 65.1% (28/43) of isolates assigned to known clonal complex were grouped into one of these four CCs.

As the different production systems were processed in specific slaughtering plants, grouping the neck skin isolates for site of collection (Table 5.15) was used to point out if specific genotypes were circulating in the different plants among the neck skin samples. ST-257 complex was the only clonal complex reported in all three slaughtering plants.

	Clonal complex									
	21	**45**	**257**	**353**	**354**	**443**	**464**	**661**	**828**	**UI**
Standard System (Plant A)		1	5	1	3		3		2	2
Freedom Indoor (Plant A)				1			3		2	1
Improved Welfare System (Plant B)	2		1	3			1	2		3
Free Range (Plant C)	4	7	1			1				
Whole population	6	8	7	5	3	1	7	2	4	6

Table 5.15: Frequency and distribution of neck skin clonal complexes among production systems and site of processing (UI unassigned isolate)

5.3.3. Breast meat and liver isolates

The distribution of STs in breast meat samples is shown in Figure 5.6. Within the *C. jejuni* population, that included 12 isolates, ST257 was the most common sequence type reported (3 isolates). Within the *C. coli* isolates ST827 (2 isolates) was the only sequence type reported. The 11 STs reported were grouped into 8 CCs. Within the *C. jejuni* population the largest clonal complex was found to be ST-257 (consisting of 4 isolates). Within the *C. coli* population the two STs reported (both ST 827) were grouped into the ST-828 clonal complex. The only liver isolate genotyped was a ST464 belonging to the ST-464 complex.

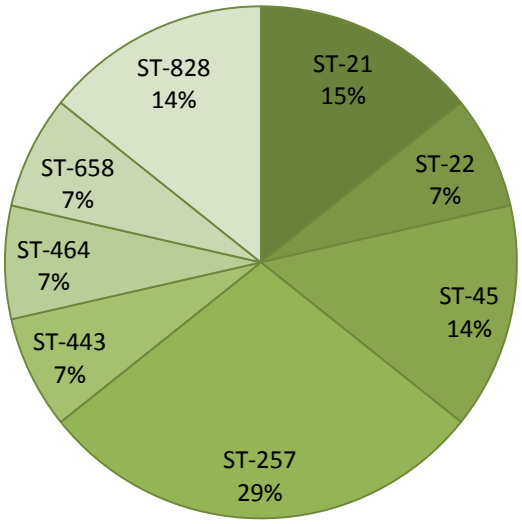

Figure 5.6: Distribution of C. jejuni and C. coli clonal complexes among breast meat isolates

5.3.4. Multiple strains within the batch

As reported in other studies, it was evident that in all production systems, most of the flocks were infected with multiple strains and mixed colonisation with *C. jejuni* and *C. coli* occurred (Jacobs-Reitsma, van de Giessen et al. 1995; Shreeve, Toszeghy et al. 2000; Bull, Allen et al. 2006; Ragimbeau, Schneider et al. 2008; Colles, McCarthy et al. 2010; Mullner, Collins-Emerson et al. 2010). A maximum of six different caecal isolates were genotyped from a distinct batch and a maximum of four different strains were observed. Colonisation with a single dominant strain as well occurred among all production systems. A maximum of 5 identical caecal isolates was reported in a Standard batch.

5.3.5. Diversity of Campylobacter strains on caeca and matching carcasses and breast meat samples

In 19 different *Campylobacter* colonised batches, isolates were recovered from both caecal and neck samples. We observed that 56% of the STs obtained from carcasses matched sequence types found in the caecal samples. On the other hand, within the six isolates collected from breast meat samples belonging to *Campylobacter* positive batches, only one isolate matched the sequence type reported in the caeca.

Ten isolates (4 from neck skin samples and 6 from breast meat) were obtained from *Campylobacter* negative batches. Among those isolates, ST-45 (50%) and ST-257 (25%) were the major clonal complexes identified.

5.3.6. Farm genotypes carriage

Two flocks coming from the same Freedom Food farm were sampled at slaughter 12 months apart. Eight isolates (6 from caeca and two from the neck skin samples) were collected in October 2010 and 8 isolates (4 from caeca, 3 from neck skin and 1 from breast meat samples) were collected in September 2011. No overlapping of STs was reported. In October 2010, all isolates from the caeca were *C. coli*; in September 2011 only *C. jejuni* was reported within the isolates genotyped.

On two different occasions, consecutive flocks from the same farm were sampled. In the first case, 6 isolates (3 caeca and 3 neck skin) from a Freedom Food free range farm were collected in February 2011, and 3 more isolates (2 from caeca and one from neck skin samples) were retained from the consecutive flock slaughtered two months later. The two flocks carried different genotypes. In the second case, two consecutive flocks from the same Improved Welfare farm were sampled at slaughter in Feb 2011 and in Apr 2011. One caecal isolate was genotyped from each flock and same sequence type ST573 (ST-573 complex) was observed.

Clonal complex	Production systems				Sample types				Seasons			
	ST	FI	IW	FR	Caeca	Neck skin	Breast meat	Liver	Winter	Spring	Summer	Autumn
ST-45 (19.9)	2 (4.1)	2 (7.40)	6 (16.2)	17 (60.7)	17 (22.1)	8 (16.3)	2 (14.3)	0	18 (50)	6 (26.1)	0	3 (4.6)
ST-828 (14.2)	10 (20.4)	5 (18.5)	5 (13.5)	0	14 (18.2)	4 (8.16)	2 (14.3)	0	6 (16.7)	0	0	14(21.5)
ST-464 (12.8)	9 (18.4)	8 (29.6)	1 (2.7)	0	9 (11.7)	7 (14.3)	1 (7.1)	1 (100)	1 (2.8)	0	8 (47.1)	9 (13.8)
ST-257 (10.6)	11 (22.4)	0	3 (8.1)	1 (3.6)	4 (5.2)	7 (14.3)	4 (28.6)	0	5 (13.9)	1 (4.3)	2 (11.8)	7 (10.8)
ST-21 (8.5)	1 (2)	1 (3.7)	2 (5.4)	8 (28.6)	4 (5.2)	6 (12.2)	2 (14.3)	0	0	10(43.4)	1 (5.9)	1 (1.5)

Table 5.7: Number and prevalence (%) of major C. jejuni and C. coli clonal complexes found among the different production systems, samples types and seasons from the 141 poultry isolates

5.4. DISCUSSION

A total of 141 *Campylobacter* isolates were successfully typed by MLST. As broadly reported in other studies, considerable genetic diversity in *Campylobacter* genotypes was identified among the isolates analysed and the three most reported sequence types (ST464, ST45 and ST257) covered only 27.7% of our dataset. The five predominant clonal complexes identified in this study, ST-45, ST-828, ST-464, ST-257 and ST-21, grouping 66% of all isolates, have been described in several studies as the most reported among the broiler populations and they were all previously described amongst retail chicken isolates (Colles, Jones et al. 2003; Karenlampi, Rautelin et al. 2007; de Haan, Kivisto et al. 2010). ST-45, together with ST-21 and ST-257 complexes, were also reported internationally as the most frequently reported *C. jejuni* genotypes in human disease, with ST-828 being the most common *C. coli* identified in the human cases (Dingle, Colles et al. 2001; Karenlampi, Rautelin et al. 2007; Wilson, Gabriel et al. 2008; Sheppard, Dallas et al. 2009; de Haan, Kivisto et al. 2010; Wirz, Overesch et al. 2010). It was this overlapping, based on genetic similarity, between human strains and the isolates dominant among the broilers population that confirmed the initial findings reported in most case control studies (Hopkins and Scott 1983; Harris, Weiss et al. 1986) that included chicken consumption as the major risk factor for human campylobacteriosis (Dingle, Colles et al. 2002; Manning, Dowson et al. 2003; Wilson, Gabriel et al. 2008; Sheppard, Dallas et al. 2009). The ability to attribute cases of food-borne disease to animal is a fundamental step for the successful prioritisation and implementation of effective food safety control measures into the food chain to reduce the incidence of human infections.

Our study is the first to describe *C. jejuni* and *C. coli* population within different commercial production systems. The aim of this molecular based project was to investigate a possible association of certain *Campylobacter* strains among the *C. jejuni* and *C. coli* population with a distinctive production system, and to potentially identify a specific reservoir for human *Campylobacter* infection across different broiler production systems. Nevertheless, there was a considerable variation in sequence types among the production systems, and genetic diversity was confirmed in all commercial systems, given the number of isolates analysed and the diversity of the *Campylobacter* population, it was not possible to estimate a particular contribution of a specific production system to human infection. In all commercial production systems examined, more than 50% of the isolates collected were grouped into the five predominant clonal complexes reported among the broiler populations worldwide. All production systems could be included as potential sources of *Campylobacter* infection in humans.

In contrast to other studies that reported a higher diversity of strains in free range flocks because of their greater exposure to the outdoor environment and therefore to potential contamination sources during the rearing period, in this study a larger number of distinct clonal complexes was observed among the Improved Welfare and the Standard systems. As well, in our Freedom Food free range flocks, in contrast to the findings reported by other authors, a higher prevalence of *C. coli* was not observed (El-Shibiny, Connerton et al. 2007; Colles, Jones et al. 2008; Colles, McCarthy et al. 2010; Colles and Maiden 2012), and no *C. coli* species were found amongst the isolates collected from the free range population. This could be related to the younger age at slaughter (an average of 54 days) of our Freedom Food free range population, as a

switch into a higher prevalence of *C. coli* compared to *C. jejuni* is mainly seen in older birds (El-Shibiny, Connerton et al. 2005; Allen, Ridley et al. 2011).

Coinciding with previous studies that used a variety of molecular approaches, the comparison of the paired caeca and neck skin samples from the same batch, indicated that the majority of *Campylobacter* strains found on carcasses appears to originate from the slaughtered flock itself (Rivoal, Denis et al. 1999; Klein, Beckmann et al. 2007; Normand, Boulianne et al. 2008; Elvers, Morris et al. 2011). This finding gave strong evidence for the importance of addressing the risk of carcass contamination due to faecal spillage during processing. Minimal variation in carcass size within the slaughtering batch, use of evisceration machines properly adapted to the carcasses' size, decreasing the line speed and even manual evisceration are all measures that, if put in place in the slaughtering plant, can reduce viscera rupture leading to intestinal content release. However, considering the cost and limitation to implement good evisceration practices in some processing plants, intervention at farm level to prevent flock colonisation could have a better effect in reducing *Campylobacter* contamination in the final products.

Despite the fact that most strains isolated from the broiler carcasses were similar to those reported in the corresponding caecal samples, diverse sequence types observed in the final products were not found in caecal samples belonging to the same batch. Even so, it could be due to the fact that it was not possible to catch completely the full diversity in the batch because of the limited number of isolates collected, the lack of overlap between strains from caeca and carcasses/breast meat samples could indicate that cross-contamination from different sources other than caeca colonisation itself,

can occur in the final products (Peyrat, Soumet et al. 2008). Stronger evidence for the potential role of processing operations and slaughterhouse environment as important sources of contamination emerged when carcasses from *Campylobacter* negative flocks tested positive after processing (Elvers, Morris et al. 2011). Transport crates, processing machines, working surfaces, workers, cutting equipment and packaging material are all implicated as possible sources of contamination of broiler carcasses from *Campylobacter* negative flocks (Peyrat, Soumet et al. 2008).

Some studies assumed that, at slaughter, carcasses from a *Campylobacter* negative flock would be cross-contaminated primarily with the strains originating from the intestines of the previously processed positive flock (Miwa, Takegahara et al. 2003). However, other studies showed instead that the neck skins of negative flock that was preceded by positive flock, were cross-contaminated by strains which did not originate from the predominant strains colonising the caeca of the flock slaughtered immediately before (Elvers, Morris et al. 2011). In this study no information on *Campylobacter* status of the previous flocks was available. Also no isolates belonging to *Campylobacter* negative flock, processed first during the working day, were genotyped and thus it was not possible to speculate further on the specific role of the slaughterhouse environment in carcass contamination.

With the current UK broilers production, the largest processing plants have a continuous cycle of production of 16-20 hours/per day. Slaughtering operations are not interrupted for any cleaning during the working day and a final deep cleaning is carried out only at the end of the operations. To use molecular tools to investigate the source and dynamic of contaminations of the final products within the processing

plant, it is important to plan preventive and corrective actions to reduce the level of contamination in the final products. Identification of a build-up of *Campylobacter* contamination during the working day in the abattoir environment, leading to cross-contamination of final meat products could drive Regulatory Bodies and Policy Makers to force regular cleaning during the operations or to implement the logistic slaughter although these corrective measures would have a significant financial impact on the poultry industry (Rasschaert, Houf et al. 2006).

It was interesting to observe that not all dominant strains found in the caeca were then recovered from carcasses at the end of the processing line, with some genotypes never found in the final products despite being present in the caecal samples. These changes in strain predominance in the end products may reflect the inability of some of them to survive during the processing with the more resistant ones being more likely to be found on the final products (Peyrat, Soumet et al. 2008; Wirz, Overesch et al. 2010). Different studies have been carried out on the genetic diversity of *Campylobacter* strains at different sampling points through the processing line. It was observed that the ability of the bacteria to resist environmental stressors (e.g. washing operations, chilling conditions, chlorine dioxide, oxygen) that occur during processing changed according to the genotypes, with specific types being able to survive the complete processing stages at poultry abattoir whereas others were never detectable at the end of the chilling process (Newell, Shreeve et al. 2001; Alter, Gaull et al. 2005). Further work is required on the survival of individual *Campylobacter* strains during processing and how these 'more resistant' strains can affect the diversity of contamination in the end product, as it is well known that although *Campylobacter*

cannot grow outside the host, it can survive for considerable periods in the processing plant's environment (Alter and Scherer 2006; Peyrat, Soumet et al. 2008).

In this study, strains belonging to ST-45 and ST-257 complexes appeared more frequently in the final products and did not match with the main strains reported in the caecal content of birds belonging to the same batch. Considering also that strains from those two clonal complexes were also the most reported in neck skin and breast meat samples associated with *Campylobacter* negative flocks, we could consider that strains belonging to those clonal complexes may have a better resistance to the environmental stressors that occur during processing and potentially have higher ability to cross-contaminate the carcasses. In particular the ST-257 complex showed a higher tendency to survive in the slaughterhouse environment: genotypes grouped in this clonal complex were the most commonly reported in breast meat samples and the second most common on carcasses despite being rarely detected in caecal samples (only 5% of prevalence). ST-257 was also the only clonal complex reported in all three slaughtering plants and in all seasons. The above is not surprising as ST-257 complex was reported in several works as the most common in retail chicken (Wilson, Gabriel et al. 2008; Sheppard, Dallas et al. 2009).

In addition to the above findings, a strain belonging to the ST-257 complex (ST3444) was also the only isolate successfully genotyped among the isolates collected from the deep muscle. The same strain was used under experimental conditions in a study carried out to investigate the extra-intestinal spread of *Campylobacter* strains in birds infected orally at 21 days of age with a suspension of 10^5 *Campylobacter* cells in 200µl of Mueller-Hinton broth. Results from this study showed that although a relatively rare

event, ST3444 has potential for invasion of edible muscle tissue in experimental system (Personal communication from the Zoonotic Infection of People, Pigs and Poultry Research Group).

With the number of isolates analysed in consecutive flocks, it was not possible to establish a clear conclusion on the carryover of *Campylobacter* subtypes between broiler flocks at farm level. However, strain persistence in the environment leading to flock colonisation from the same sources, rather than failures in the cleaning and disinfection operations and persistence of *Campylobacter* inside the broiler houses during the down-time, was considered when farm genotypes carriage was observed. It has been reported in different studies that the final cleaning and disinfection operations at the end of each production cycle are adequate to eliminate *Campylobacter* contamination inside the poultry house (Berndtson, Danielsson-Tham et al. 1996; Evans and Sayers 2000; Shreeve, Toszeghy et al. 2002; Newell and Fearnley 2003).

As seen also in other molecular studies, the sensitivity is limited by the number of isolates analysed. However, due to time constraints and the cost of MLST techniques, the number of isolates could not be increased, and conclusions made need to take into consideration the limitation in the number of isolates investigated. In particular, the workload and the cost implication of re-growing frozen isolates were significant and some isolates were no longer viable. A larger amount of incomplete typing was found among the frozen isolates, compared to the results achieved when working with fresh isolate.

CHAPTER 6
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Food-borne diseases are currently considered as one of the major public health problems in the world, and among food-borne pathogens *Campylobacter* is recognised as the most important organism in terms of disease burden as measured by the number of cases, hospitalisations and cost of illness (Adak, Long et al. 2002; Adak, Meakins et al. 2005). Although all food animals can carry *Campylobacter*, chickens pose the greatest risk for human infection, since most of the world's commercial poultry production is *Campylobacter* positive at slaughter and furthermore due to the large quantities of chicken meat consumed (Humphrey et al. 2007).

The overall aim of this thesis was to investigate the association of different poultry production methods with *Campylobacter* prevalence and transmission in the broiler meat food chain, with the further objective to link the flock *Campylobacter* status to the risk of contamination on the consumer's plate. The analyses carried out have not considered the potential effect of individual farm/flock parameters, thinning procedures, and other slaughterhouse parameters on *Campylobacter* infection, as a consequence the results obtained in our study reflected all the combined effects that were inextricably related to each broiler production system within the specific Company.

The cross sectional study (Chapter 2) showed a high prevalence of colonised batches at slaughter in the broiler population investigated. All production systems studied appeared to be vulnerable to colonisation by *Campylobacter*. Interestingly, it was

found that the prevalence of flock colonisation differed according to the production system. More particularly these results suggest that birds reared indoors under higher welfare standards with reduced stocking density with a slower growing breed (Hubbard J57/Freedom Food Indoor) had a reduced prevalence of *Campylobacter*, compared to the standard fast growing breed (Ross 308/Improved Welfare system), when grown at the same stocking density and under the same environmental conditions. However, as analysed in Chapter 4, specific farm parameters and farm practices such as flock size and the process of flock thinning, could have played an important role in the higher *Campylobacter* incidence reported in the Improved Welfare system, which merit further investigation.

It is necessary to determine which factors linked to a particular production system have a big impact on flock colonisation. Furthermore it is essential to identify if it is the bird type or the environmental conditions that are playing the major role in *Campylobacter* colonisation. Bearing in mind that the British customers are more preoccupied when buying meat with the welfare of the animals than the potential public health risks, such as pesticides, food additives and pathogens such as *Campylobacter* (EFSA 2010e), it is important to make them aware that a higher welfare "label" does not necessarily produce healthier and happier chickens.

Throughout our studies (Chapters 2, 3 and 4) it emerged that the level of contamination on the final products is strongly associated with flock colonisation during the rearing period. This finding was in accordance to previous studies that identified the colonisation of the live flock as the main source of *Campylobacter* contamination during the slaughter operations (Jeffrey, Tonooka et al. 2001; Lindblad, Hansson et al. 2006; Allen, Bull et al. 2007; Reich, Atanassova et al. 2008; Hansson,

Engvall et al. 2010). Consequently, the main objective to reduce the risk of human infection should be to deliver at slaughter *Campylobacter*-free animals. However, to prevent or to reduce the number of colonised flocks is challenging as *Campylobacter* is ubiquitous in the environment and currently there are no reliable means for preventing them from entering into poultry houses. Furthermore the understanding of this pathogen is not as extensive as other major food-borne bacteria such as *E. coli* and *Salmonella*, and more studies are needed to investigate which mechanisms are influencing colonisation of flocks.

As identified in our longitudinal study (Chapter 4), *Campylobacter* colonisation can occur at all stages during the farm growing period. It emerged that it is difficult to single out the specific sources responsible for flock colonisation during the rearing period and to understand why some farms using the same production system were able to rear *Campylobacter*-free chickens and others were not. Furthermore it appears challenging to grasp the reasons why, in the same farm, consecutive production cycles could differ in the *Campylobacter* infection rate. A more systematic analysis of the data using multilevel modelling may clarify the effect of some of the risk factors for which information was collected in this study and univariable analyse has not been able to provide conclusive evidence or eliminate confounders. Furthermore, a more thorough flock investigation, by setting up more regular and systematic sampling procedures during the rearing period, is necessary to identify the sources of entry of this pathogen into the broiler houses. As experienced during the field work, a better interpretation of the laboratory data would be achieved if closer field collaboration would take place between the researcher, the producer and its farm operatives. By spending time in the

farms, observing the daily practices and talking with the key actors at operative level would allow a greater transparency in the correlation between cause and effect.

One of the most recognised measures to prevent flock colonisation is to ensure that good bio-security safety measures are maintained throughout the whole rearing period (Hald, Wedderkopp et al. 2000; Humphrey, O'Brien et al. 2007). Bearing this in mind, the fact that the UK poultry industry introduced a new on-farm bio-security policy as part of the Red Tractor Assurance Chicken Production scheme in April 2011, gave the FSA the confidence that the predicted target set in 2010 to reduce the level of *Campylobacter* contamination in poultry carcasses, would be met by the end of 2015 (FSA 2010). However, interim data reviewed in September 2013, showed clearly that the level of carcass contamination in the UK was still dramatically high and considerably below target (FSA 2013). The lack of improvements could be explained by the fact that high bio-security standards are difficult to be followed consistently and effectively during the daily routines. However, the importance of bio-security must not be overestimated, as factors other than environmental contamination, such as the chicken breed, immune system, hormones produced in response to the stress and chicken health have also an essential part to play in flock colonisation (Humphrey 2006; Cogan, Thomas et al. 2007; Colles, Jones et al. 2008). Without taking into account these other factors, the delivery at the processing plant of *Campylobacter* negative free range flocks cannot be explained, since free range chickens are subject to a greater bio-security risk when they are released into the range.

Throughout the cross sectional and longitudinal studies (Chapter 2 and Chapter 4) it emerged that when *Campylobacter* entered into a poultry flock all chickens became colonised and stayed colonised up to the time of slaughter. From the above findings

we can bring to light the importance of keeping *Campylobacter* out of the flocks, as it appears impossible to limit the spread of infection between the animals during the rearing period (Berndtson, Danielsson-Tham et al. 1996; Shreeve, Toszeghy et al. 2000). Inside the broiler house, communal sources of eating and drinking, together with the normal coprophagic activity of broilers, facilitate the spread of *Campylobacter* among the flock (Herman, Heyndrickx et al. 2003; Messens, Herman et al. 2009).

A better understanding of the interactions between the bacteria and the chicken host, would be required to develop different intervention strategies at farm level. Reducing the number of bacteria in the chicken flocks may be possible with combinations of vaccination, bacteriophage or bacteriocin treatments, feed additives and competitive exclusion agents. However, these novel methods to prevent or reduce flock colonisation are still far from being used commercially and more investigations and investments are needed (Aho, Nuotio et al. 1992; Hakkinen and Schneitz 1999; Chen and Stern 2001; Chaveerach, Keuzenkamp et al. 2002; Carrillo, Atterbury et al. 2005; Stern, Svetoch et al. 2005; Wagenaar, Van Bergen et al. 2005; de Zoete, van Putten et al. 2007; Havelaar, Mangen et al. 2007).

In the UK there are no legislative requirements to enforce that poultry producers deliver Campylobacter-free flocks at slaughter, and since Campylobacter does not appear to cause disease in the chicken host itself, only strategies that are low cost and are efficient in preventing and/or reducing the flock colonisation would be taken into consideration by the poultry industry. As discussed in Chapter 4, measures such as elimination of the partial depopulation of the flocks, limitation of transport time and better management of the crates have big cost implications, which the poultry industry is not ready to take on board considering its low profit margins and the fact that to

control Campylobacter is not directly related to flock production. However, the findings reported in our studies (Chapters 2 and 4), showed that *Campylobacter* colonisation is positively associated with welfare associated pathologies. Nevertheless further research is needed to investigate the mechanisms that link *Campylobacter* infection with the health and the welfare of the birds our results indicate that there is a combined benefit for public health and animal welfare in the control of *Campylobacter* infection.

It is well known that birds with severe contact dermatitis lesions can suffer lameness and show slower weight gain due to pain-induced reduction in appetite (Martland 1985; McGeown, Danbury et al. 1999; Shepherd and Fairchild 2010; RSPCA 2011). In addition to the above welfare issues, for quality reasons birds with contact dermatitis such as breast blisters and hock burns cannot be sold as whole birds, hence impacting on the profitability of the flock. Furthermore, in the last decade, the demand from Asian countries, such as China and Hong Kong, for chicken feet has dramatically risen. This export market has turned chicken feet (paws) into the third most lucrative part of the chicken, after breasts and wings. As a result of the above, it is clear that the downgrading and the condemnation of chicken feet due to pododermatitis, seriously reduces the companies' profit margins (Shepherd and Fairchild 2010). Consequently, if a direct link between *Campylobacter* and contact dermatitis is proven, it would be in the best interest of the poultry producers to invest more in preventive measures to reduce flocks colonisation because of the threat to public health, but also for welfare and economic reasons.

Further studies are needed to determine the role played by *Campylobacter* on gut health and the impact on the chicken immune status. Bearing in mind the limitation of

this study, in regard to the fact that clustering and potential confounding were not considered in the analysis, it appears that it is mainly in fast growing birds that the presence of *Campylobacter* is associated with the incidence of hock marks and pododermatitis. Immunological differences between 'slow' and 'fast' broilers under *Campylobacter* invasion and differences in gut health between different breeds are currently being investigated at Liverpool University. However, for the limitation said above and the current knowledge of difference in bird types, at this stage the use of slower growing birds cannot be enforced to improve the welfare and the profitability until more data is widely available (Humphrey 2013).

Since the presence of *Campylobacter* in the UK poultry flocks will almost certainly remain a major public health concern, it is conceivable that the FSA and DEFRA would set statutory requirements for the monitoring and control of *Campylobacter*, as was successfully done in 2009 with the implementation of the UK National Control Programme to control *Salmonella* in broiler flocks, though of course this was mandatory due to the EU legislation on control of zoonoses and had precedent in layer flocks (DEFRA 2009). Such potential legal requirements would have to be met by the poultry producers if they want to keep producing fresh poultry products. One of the possible repercussions of the application of these legal requirements would be a significant cost increase for the poultry industry, which would be passed to the final customer, or if cheap chicken remains the priority to a decline in the UK production leading to increased reliance on imported poultry meat.

As reported in other national and European studies, this research shows the poultry processing industry has to cope with *Campylobacter* positive broilers as starting material. During the processing, this fact adds extra pressure on the food safety

management system which is primarily based on hygienic measures and implementation of the compulsory HACCP system as per EU Regulation 852/2004 (EFSA 2011). Through our research, one of the most recurrent findings was the cross-contamination of carcasses during the automated process, and the difficulty to contain the spread of the pathogen during the operations when *Campylobacter* positive flocks were processed (Berrang, Buhr et al. 2001).

In Chapter 3, we analysed how *Campylobacter* contamination levels on carcasses vary at different stages of the processing line, and how it is challenging to reduce product contamination to a safe level. We observed that steps such as final washing and chilling, which have been set to reduce the bacteria contamination during the production, failed to reach their intended purpose if the flocks arrived at slaughter highly colonised with the pathogen. Furthermore, the data showed that the final washing increased the level of contamination on carcasses. This finding is problematic because it raises the question of the efficacy of the final wash during the processing. Processing plants need to set final washing efficiently, according to the size of the birds, throughput processed and line speed. As reported by others, the chilling step appeared to give a better response in the reduction of *Campylobacter* contamination. However, despite the fact that the number of the bacteria decreased along the meat production, the level of contaminated carcasses leaving the air-chilling with high number of bacteria was still significant and presented an unacceptably high public health risk (Nauta, Hill et al. 2009; Swart, Mangen et al. 2013).

Several options are under investigation to act as intervention measures in the poultry processing line to reduce the number of *Campylobacter* on poultry meat, and some chemicals such as lactic acid, chlorine dioxide, peroxyacetic acid and acidified sodium

are already being used in other larger poultry producer countries such as the USA and Brazil to tackle contamination. However, EC Regulation 853/2004 allows decontamination treatments to be used only if the substance is shown to be safe, and until now insufficient data has been submitted to EFSA to justify approval of any chemical treatment. Consequently, no chemical decontamination measures are currently authorised in the EU for use in raw meat. Other retail interventions such as different modified atmosphere packaging are still under investigation. However, since in Europe, the rate of *Campylobacter* human infection is rising each year (EFSA 2012), it is expected that at European level *Campylobacter* legal requirements in meat products would be implemented, similar to the microbiological criteria set in the EU Regulation 2073/2005 for certain food-borne pathogens such as *Salmonella* and *Listeria monocytogenes*.

As it is well recognised that all food animals can carry high number of *Campylobacter* in their intestines, it is important to highlight which are the main factors that give lower *Campylobacter* prevalence in red meat compared to the chicken products (Ghafir, China et al. 2007; Little, Richardson et al. 2008). Apart from the different colonisation levels during the farming period (Kwan, Birtles et al. 2008), the main reasons that can be attributed to this lower prevalence are the different hygiene practices followed during the meat processing that prevent the spread of cross-contamination. For example in red meat plants, the speed of the production line is reduced compared to the completely automated systems used in the poultry processing plants. In addition, in red meat plants, intestine removal is carried out manually, ensuring that the whole intestinal pack is not opened during the evisceration, as a result the risk of cross-contamination is kept to a minimum. When

contamination occurs, it can just be removed by trimming as the use of water to eliminate visible contaminations is not allowed in red meat plants. Furthermore manual equipment used during processing, such as knives and saws, are easily and regularly washed and sterilised. Moreover the fact that, with the exception of pork, in red meat products the skin is removed before reaching the final consumer, is eliminating a further hazard when red meat is consumed. Finally, red meat carcasses are also exposed for longer periods of time to the dry chilling conditions. This has an impact on the final number of *Campylobacter* on carcasses, as the pathogen appears very sensitive to dryness (Berrang and Northcutt 2005; Alter and Scherer 2006; Murphy, Carroll et al. 2006; Silva, Leite et al. 2011) .

As seen in the longitudinal study (Chapter 4), the internal contamination of edible tissues was reported. This poses a major public health threat, as the bacteria found within the muscle tissue are better able to survive cooking. Further research is needed to investigate factors such as mucosal damage, infection with other pathogens and host factors, causing extra-intestinal spread of *Campylobacter*.

The integration of molecular tools into epidemiological studies to investigate the origin of human campylobacteriosis has played a major part on the understanding of contributions of different reservoirs to human infection (Colles and Maiden 2012). In this research, Chapter 5, MLST was used to determine the diversity and *Campylobacter* population structure among the four different commercial production systems. A high genetic diversity was observed among the population studied. However, five major clonal complexes ST-45, ST-828, ST-464, ST-257 and ST-21 were predominant in our dataset (66% of all isolates), and they have been described in several studies as the CCs most reported among the broiler populations in different part of the world and among

human isolates (Colles, Jones et al. 2003; Karenlampi, Rautelin et al. 2007; de Haan, Kivisto et al. 2010; Griekspoor, Engvall et al. 2010).

We investigated if there was a contribution of a specific production system to the human disease burden. Nevertheless, there was a considerable variation in sequence types among the 4 groups investigated, in all commercial production systems more than 50% of the isolates collected were grouped into the five predominant CCs reported among the worldwide broiler populations. With the number of isolates analysed and the diversity of the *Campylobacter* population, it was not possible to estimate a particular contribution of a specific production system to human infection. Molecular investigation showed that all production systems could be included as potential sources of *Campylobacter* infection in humans.

As reported in other studies, *Campylobacter* species identification highlighted a higher *C. jejuni* prevalence compared to *C. coli* among our isolates (EFSA 2010a; Bardoňa, Kolářb et al. 2011; Jorgensen, Ellis-Iversen et al. 2011), and both species showed to have a similar ability to survive during the processing. However, changes in strain predominance in the end products were observed. This may reflect the enhanced ability of some genotypes to resist environmental stressors, such as washing operations, chilling conditions, chlorine dioxide and oxygen that occur during processing (Callicott, Hargardottir et al. 2008; Peyrat, Soumet et al. 2008; Hunter, Berrang et al. 2009; Wirz, Overesch et al. 2010). In this data set, strains belonging to the ST-257 complex showed a higher tendency to survive in the slaughterhouse environment, and also potential for invasion of edible muscle tissue. Sequencing of mRNA transcripts (RNAseq) and whole-genome sequencing (WGS) approaches are providing promising insights into how this pathogen responds and adapts to the host

and its environment (Colles and Maiden 2012; Taveirne, Theriot et al. 2013). However, further works are required to identify how different strains respond differently to adverse environmental conditions, and to understand which mechanisms are involved in the bacteria colonisation of its natural hosts (Alter and Scherer 2006).

Due to the common extent of this pathogen in food and its impact on human health, it is necessary for the Government bodies, food producers and retailers, to raise consumers' awareness of the *Campylobacter* issue. Particularly the consumers must be made aware of how to manage the risk appropriately during the food preparation. Currently in recognition of this issue, some UK restaurants have already started to make their guests sign a consent form to absolve their responsibility when blue/rare meat is ordered.

6.1 Summary of future work

Should more funding be available in the near future, to further investigate the causes and effects of *Campylobacter* on broilers, below is a summary of the areas singled out throughout this thesis that need further research.

1. The timing and the sources of entry of the pathogens into the broiler houses.
2. The interaction between animal health, welfare, stress, farm management and *Campylobacter*: to identify if it is the bird type or the environmental conditions that are playing the major role in *Campylobacter* colonisation.
3. Factors such as mucosal damage, co-infection with other pathogens and other host factors causing the extra-intestinal spread of *Campylobacter* into edible tissue.

4. The impact of measures such as freezing and cooking in the reduction of the deep tissue contamination.
5. Mechanisms that are involved in the different strains' responses to the environmental conditions throughout the food chain.

APPENDIX 1

FOOD CHAIN INFORMATION FOR POULTRY	Reference No:

Part 1 – Information about Producer and the Veterinary Surgeon

A. Producer details

Name of Producer and his position e.g. keeper, owner etc..,	
Address of Producer (farm)	Post code
County parish Holding Number	Tel no Fax no
Email address	

Are you a member of any assurance scheme? **YES/NO**

If **YES**

Name of assurance scheme		Membership number	

B. Veterinary surgeon & practice details

Name of Veterinary Surgeon	

Name and address of Veterinary Practice (if different)	Post code

Email address (if known)		Tel. number	

*__Destination:__ Slaughterhouse Name /Approval Number (if known):

Part 2 – Information about poultry being sent for slaughter

	House 1	House 2	House 3	House 4
Species				
Breed or hybrid (broilers only)				
Age				
Production type [free range, housed or organic etc.]				
No of birds				
Batch identification reference e.g. trailer / shed no.				
Proposed slaughter date				
*Maximum stocking density (broilers only)				
*Mortality % at 14 days				
Mortality % to date or for broilers only: Cumulative daily mortality rate				

Name of medication prescribed (include vaccines and preventative medicines – coccidiostats)	Houses	Date withdrawn	Have the withdrawal periods been observed?

Has any disease been diagnosed in this shed/house? **YES/NO**

If **YES** give details

Has the mortality rate been High for a reason other than disease? **YES/NO**

If **YES** give details

Is this flock required to be tested under the requirements of the Salmonella National Control Programme (NCP)? **YES/NO**

If Not Exempted **–** Please provide**:**

Date of test

Result of test:

(Negative or Positive, including Salmonella type isolated if positive)

Have any other tests been carried out on the flock for any agents with the potential to cause food-borne disease in humans? **YES/NO**

If **YES**

Agent tested for

Date & Result of test

Part 3 – Disease history of the holding

Is the holding under any statutory or voluntary restrictions? **YES/NO**

If **YES** what restrictions apply?

Provide the following information ONLY if previous consignments were sent to a different slaughterhouse

In 2 previous consignments from this (these) house (s)/shed (s) please record the rejection rate

Reason for rejection

% Rejected

Reason for rejection		% Rejected	

To be signed by the person responsible for completing Parts 1, 2 & 3

Signature of Producer		Date		Time	

Part 4 – Slaughterhouse operator's check and comments

I accept these birds for slaughter for human consumption

Signature		Date		Time	

Comments	

Part 5 – Official or Approved Veterinarian's check and comments

FCI checked

Signature		Date		Time	

Comments	

NB: * denotes optional requirements but see accompanying guidance note.

GUIDANCE FOR COMPLETION OF FOOD CHAIN INFORMATION (FCI) FOR POULTRY

Timing

FCI is required to be supplied at least 24 hours before the arrival of animals at slaughterhouse, except where ante mortem inspection is done at the farm. In this case the FCI and veterinary ante mortem declaration is to accompany the animals to which they relate.

(Regulation (EC) No 853/2004, Annex II, Section III and Regulation (EC) No 854/2004, Annex I, Section I, Chapter II A and Section II, Chapter II.)

Part 1, 2 and 3 to be completed by the producer.

Part 4 to be completed by the slaughterhouse operator.

Part 5 to be completed by the Official or Approved Veterinarian.

PART 1 - INFORMATION ABOUT PRODUCER AND THE VETERINARY SURGEON

The producer is the person in charge of birds being sent for slaughter. This may be the owner of birds, farm manager, keeper or grower. The email address provided should be that to which you wish the results of the inspection activities to be sent.

PART 2 - INFORMATION ABOUT POULTRY BEING SENT FOR SLAUGHTER

Provide only details relevant for birds from a particular house or shed that are being sent for slaughter.

EU Broiler Directive (2007/43/EC)

This EU Directive lays down minimum rules for the protection of chickens kept for meat production. It applies to conventionally reared meat chickens (broilers) on holdings with 500 or more birds.

Under this legislation, keepers who stock above 33kg/m^2 will need to provide details of breed/ hybrid line and the most recent cumulative daily mortality rate for each house . In addition we request that the maximum stocking density of the flock is included to this form. We would encourage all keepers, regardless of flock stocking density, to provide the information requested in this form to assist in on-going data collection and evaluation.

The Defra website has information and guidance on the requirements of the Directive at http://www.defra.gov.uk/foodfarm/farmanimal/welfare/onfarm/meatchks-newrules.htm including how to calculate cumulative daily mortality rate.

Mortality % at 14 days for breeders/layers to be provided if known.

Details of medicines given should only cover those where withdrawal period is greater than zero. For breeders and layers the period covered is to include the last six weeks as a minimum.

Salmonella National Control Plan (NCP)

Give details of the flock test for *Salmonella* carried out under the requirements of the *Salmonella* NCP including the results of the test.

Completion of the *Salmonella* testing details is obligatory for all poultry covered under the breeder, layer, broiler and turkey NCPs unless there is exemption for one of the specific criteria as laid out in the guidance to the NCP.

- The UK Guides to the National Control Programmes for Salmonella in breeders, layers, broilers and turkeys is available on the Defra website at http://www.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/zoonoses/ncp.htm or from your local Animal Health Office.

Also give details of any disease diagnosed or any other agents identified in the flock, if tested and results known e.g. Microbial and/or Chemical contaminants.

PART 3 - DISEASE HISTORY OF THE HOLDING

If applicable, please provide details of any disease diagnosed on your farm (e.g. Avian Influenza, Newcastle Disease) where movement restrictions have been imposed.

NOTE: The person completing Parts 1, 2 and 3 is to sign the box provided.

PART 4 - SLAUGHTERHOUSE OPERATOR'S CHECK AND COMMENTS

This check is concerned with completeness of document for obvious errors and omissions rather than making the professional evaluation of information supplied. However, when for example birds have been tested positive for Salmonellae, the slaughterhouse operator will have to note down action taken. In order to minimise cross contamination during processing the action taken may include processing the batch before the break or last in the day.

PART 5 - OFFICIAL OR APPROVED VETERINARIAN'S CHECK AND COMMENTS

As part of ante-mortem inspection, the FCI may be checked either on farm or in the slaughterhouse.

APPENDIX 2

SLAUGHTERHOUSE QUESTIONNAIRE

Factors associated with Campylobacter-positivity at slaughter to be considered in the analysis

Farms considerations

(Data to be collected from the Food Chain Information and from the Agriculture Department)

- Farm details
- Farm assurance scheme
- Flock production type
- Breed
- Numbers of houses in the farm
- Flock size
- Stocking density
- Placement date/s of chicks in the farm
- Salmonella (+ or -)
- Feed information

Slaughter batch information

(Data to be collected from the Food Chain Information and from the Agriculture Department)

- House number
- Sex of the birds
- Number of birds per house
- Age of birds per slaughtering batch
- Number of birds per slaughter batch

- Mortality
    - at 14 days
    - at 28 days
    - at 35 days
    - at 42 days
- Feed withdrawal time
- Treatments given
- Thinning procedures and numbers of catch per flock
- Time of catching
- Time of arrival of birds in the slaughterhouse
- Number of bird for crates
- Average weight of the birds
- Weather conditions *(Temperature inside and outside the lairage, to be collected from the 'lairage sheet')*
- Conditions of flocks at the arrival *(Birds health to be checked in the lairage)*
- Welfare indicators: pododermatitis and hock burns *(in % from QA sheet and observation)*
- Crates system used for the transport (Solid or open floor)
- Time start processing
- Order of slaughtering (Killing order)
- Time finish processing
- Time sampling collection

Specific slaughterhouse factors

- **Slaughterhouse details**
    - Capacity and speed line
    - Working patterns
- **Crates and modules management**
    - Type of washing system
    - Detergent, sanitizer and concentration used

- Temperature of the water
- Any swabs being carried out on crates to verify the cleaning
- Hygiene of crates and modules observed

- **Cleaning and disinfection of vehicles**
  - Type of system used
  - Detergent, sanitizer and concentration used
  - Temperature of the water
  - Any swabs being carried out on the vehicle to verify the cleaning
  - Hygiene of vehicles observed
- **Scald tanks system**
  - Is it a multiple tanks system?
  - Water temperature *(To be taken from the QA sheet)*
  - Any water recycling system in place inside the scald tanks
- **Plucking system**
  - To evaluate (in %) any escape of faecal material through the cloaca by action of the fingers pressing the abdomen
- **Evisceration system**
  - Any automatic washing system of the evisceration machines
  - % of viscera rupture observed at evisceration
  - Reaction procedure of factory staff to evisceration issues
- **Water supply**
  - Any disinfection system in place in the water supply?
  - Is chlorine being added to the water?
  - Any issues reported during the monthly sampling testing?
- **Slaughter hygiene practices**
  - Staff hygiene practices and slaughterhouse cleaning *(To be scored satisfactory/unsatisfactory after observation)*
  - Any contamination incidents observed (and reaction procedure of factory staff to contamination incidents)
  - Any break cleaning carried out? How?

- **Batch rejections (reasons for condemnation)**
  From PIA/MHI rejections record

DOA	*PERICARDITIS (total and partial rejections)*	*SEPTICAEMIC CARCASE/FEVER*
CULLS	*SKIN LESIONS*	*MACHINE DAMAGE*
ASCITES	*EMACIATED*	*UNCUT/BADLY BLED CARCASE*
OVERSCALD	*HEPATITIS (total and partial rejection)*	*WINGS/LEGS DAMAGE*
BRUISED	*PERIHEPATITIS*	*CONTAMINATION*

- **Final wash system**
  - Type of final wash
  - Pressure of the water (from QA sheet)
  - Volume of water used per bird (if known)
  - % of contaminated carcases after final washing
- **Chilling conditions**
  - Chilling system (Air chilling or water immersion chilling)
  - Time of carcases in the chiller
  - Temperature of the chiller (To be checked in the thermograph record)
  - Humidity
  - Temperature of carcasses chiller exit *(To be checked manually in 3 carcases at the exit of chiller)*

APPENDIX 3

MEAN ENUMERATION RESULTS (CFU/G) PER BATCH

Sampling	Date	Farm ID	*Farm Code*	*Neck Skin Post Chilling*	*Caeca*
1	23/08/2010	FB1	1	0.00E+00	0.00E+00
2	02/09/2010	ST1	2	3.02E+03	8.60E+07
3	29/09/2010	ST2	3	6.22E+02	0.00E+00
4	05/10/2010	ST3	4	1.24E+04	8.28E+07
5	07/10/2010	FB2	5	0.00E+00	0.00E+00
6	19/10/2010	EL1	6	Samples not collected	1.85E+08
7	19/10/2010	EL2	7	Samples not collected	7.03E+08
8	26/10/2010	FB3	8	5.03E+03	4.18E+08
9	01/11/2010	FB4	9	0.00E+00	0.00E+00
10	02/11/2010	ST4	10	9.40E+01	0.00E+00
11	07/12/2010	EL3	11	1.74E+04	4.21E+07
12	07/12/2010	EL4	12	1.29E+04	8.50E+07
13	28/01/2011	ST5	2	0.00E+00	0.00E+00
14	07/02/2011	FR1	13	1.58E+04	2.09E+07
15	07/02/2011	FR2	14	1.64E+03	1.78E+07
16	14/02/2011	ST6	15	1.31E+04	7.59E+07
17	15/02/2011	EL5	16	2.70E+03	1.42E+08
18	28/02/2011	FB5	17	0.00E+00	0.00E+00
19	28/02/2011	ST7	18	1.20E+02	0.00E+00
20	28/02/2011	ST8	19	2.03E+03	2.51E+07
21	28/02/2011	FR3	20	5.20E+02	1.96E+08
22	01/03/2011	FR4	21	0.00E+00	0.00E+00
23	10/03/2011	FR5	22	Enumeration issues	1.51E+08
24	17/03/2011	FB6	23	0.00E+00	0.00E+00
25	17/03/2011	ST9	24	8.28E+03	Enumeration Issues

Sampling	**Date**	**Farm ID**	**Farm Code**	**Neck Skin Post Chilling**	**Caeca**
26	17/03/2011	ST10	25	0.00E+00	0.00E+00
27	16/03/2011	EL6	11	1.57E+04	4.04E+07
28	17/03/2011	FR6	26	Enumeration issues	1.57E+06
29	21/03/2011	FB7	5	0.00E+00	0.00E+00
30	21/03/2011	ST11	27	Samples not collected	3.76E+07
31	21/03/2011	FR7	28	2.50E+04	1.49E+08
32	22/03/2011	FR8	28	1.67E+04	1.00E+07
33	28/03/2011	FR9	29	3.59E+03	2.84E+08
34	31/03/2011	FR10	30	0.00E+00	0.00E+00
35	05/04/2011	FR11	31	5.00E+01	0.00E+00
36	07/04/2011	EL7	16	3.12E+03	2.52E+08
37	07/04/2011	FR12	32	3.60E+02	1.68E+07
38	12/04/2011	FR13	14	2.50E+02	3.01E+07
39	14/04/2011	FR14	13	1.90E+03	3.05E+08
40	18/04/2011	FR15	33	3.92E+03	6.91E+07
41	19/04/2011	FR16	33	9.20E+02	5.52E+07
42	26/04/2011	FR17	34	4.12E+03	8.35E+07
43	09/05/2011	FR18	35	0.00E+00	0.00E+00
44	26/05/2011	FB8	36	0.00E+00	0.00E+00
45	26/05/2011	ST12	37	0.00E+00	1.09E+04
46	27/05/2011	ST13	38	1.03E+03	2.09E+07
47	07/09/2011	ST14	39	0.00E+00	0.00E+00
48	18/09/2011	ST15	40	1.40E+02	0.00E+00
49	20/09/2011	FB9	1	0.00E+00	0.00E+00
50	21/09/2011	ST16	41	2.00E+03	2.08E+08
51	22/09/2011	ST17	37	8.99E+03	7.61E+08
52	29/09/2011	FB10	8	4.70E+02	1.20E+07
53	06/10/2011	FB11	17	0.00E+01	0.00E+00

Sampling	**Date**	**Farm ID**	**Farm Code**	**Neck Skin Post Chilling**	**Caeca**
54	10/11/2011	FB12	42	1.47E+03	5.57E+08
55	08/02/2012	EL8	43	4.89E+02	8.71E+07
56	01/03/2012	FB13	42	1.23E+03	6.93E+07
57	06/03/2012	ST18	39	9.05E+03	1.28E+09
58	07/03/2012	ST19	1	0.00E+00	0.00E+00
59	09/03/2012	ST20	40	1.30E+02	2.00E+08
60	15/03/2012	ST21	37	0.00E+00	0.00E+00
61	18/03/2012	ST22	41	0.00E+00	0.00E+00
62	19/03/2012	FB14	1	0.00E+00	0.00E+00
63	21/03/2012	EL9	44	4.00E+03	5.20E+08
64	29/03/2012	FB15	8	7.36E+03	7.72E+07
65	02/04/2012	FB16	17	0.00E+00	0.00E+00
66	19/04/2012	FB17	5	0.00E+00	0.00E+00
67	23/04/2012	EL10	45	7.00E+01	4.19E+08
68	29/04/2012	ST23	39	9.30E+02	1.14E+09
69	04/05/2012	ST24	40	Samples not collected	0.00E+00
70	16/05/2012	ST25	37	2.38E+04	1.93E+08
71	17/05/2012	FB18	1	0.00E+00	0.00E+00
72	21/05/2012	EL11	46	8.33E+03	1.92E+08
73	24/05/2012	FB19	8	4.63E+03	2.72E+08
74	07.06.2012	FB20	17	0.00E+00	0.00E+00
75	21/06/2012	FB21	42	6.60E+02	2.01E+08
76	06/07/2012	ST26	41	7.80E+02	4.13E+08

(Enumeration issue: excessive contamination of plates due to lack of antibiotics in the Campylobacter growth medium)

APPENDIX 4

CONSENT FORM

Title of Research Project: Effect of different poultry production methods on *Campylobacter* incidence and transmission

Researchers: Dr Paul Wigley, Dr Eleni Michalopoulou, Dr Nicola Williams and Miss M. Camilla Brena

Please initial box

1. I confirm that I have read and have understood the information sheet dated [/ /] for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. ☐

3. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish. ☐

4. I agree to take part in the above study. ☐

Participant Name	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

The contact details of lead Researcher (Principal Investigator) are:

Dr Paul Wigley
University of Liverpool
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Chester High Road
Leahurst, Neston,
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APPENDIX 5

Miss M. Camilla Brena
DVM OV MRCVS
University of Liverpool
Faculty of Life and Health Sciences
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School of Veterinary Science
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Leahurst, Neston,
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E : mcamilla@liverpool.ac.uk
www.liv.ac.uk

Date:

Dear

You are being invited to participate in a research study on the Effect of Different Poultry Production Systems on *Campylobacter* incidence and transmission. The research is part of my PhD studies at the University of Liverpool in cooperation with a 'Poultry Company'.

Before you decide whether to participate, it is important for you to understand the purpose of my research and what it will involve. Please take time to read the following information carefully and feel free to ask us if you would like more information or if there is anything that you do not understand. We would like to stress that you do not have to accept this invitation and should only agree to take part if you want to.

Thank you for your collaboration in anticipation.

Maria Camilla Brena

Study on the Effect of Different Poultry Production Systems on *Campylobacter* incidence and transmission

I am a PhD student in the University of Liverpool, Institute of Infection and Global Health (School of Veterinary Science), and I am carrying out research on the differences in *Campylobacter* prevalence between different systems of broiler production management. For my research I am cooperating with a 'Poultry Company' who has allowed access for sample collection during further processing and provided farm contact details for Standard and Freedom Food Indoor farms in the UK.

To gather the information that I need for this research, I would like to visit your farm to collect data and to complete a questionnaire focused on farm management and bio-security practices. My observations will be concentrated on *Campylobacter* control. During my visit I would also like to collect samples from birds and litter at different stages within the production cycle. I would like to confirm to you that if there are no arrangements for you to collect your own samples I will personally carry out all farm visits, data and sample collections. All information that you share with me will only be used to inform the purpose of my research and will be treated as confidential. The principle of anonymity and confidentiality will be respected throughout my study. Individual farm results will not be available to anyone (including the 'Poultry Company') outside the immediate group of me and my supervisors. Summary results for each of the production systems will be published as part of my PhD thesis and in peer reviewed publications. Moreover, your involvement in this research is voluntary. I hope that you will be involved for at least one year, as I would like to collect data at least during two complete cycles of production, to take in consideration also the seasonality of this pathogen. Nonetheless you can choose to leave the research project at any time if you wish.

If you are unhappy at any stage, or if there is a problem, please feel free to let us know by contacting Dr Paul Wigley on 0151 7956065 and we will try to help. If you remain unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide details of the name or description of the study (so that it can be identified), the researcher(s) involved, and the details of the complaint you wish to make.

Thank you for your time and co-operation.

Maria Camilla Brena

APPENDIX 6

Farm questionnaire: Longitudinal study "from farm to fork"

Farm factors associated with *Campylobacter*-positivity to be included in the analysis

(to be completed by the principal investigator in cooperation with the farm representative)

- Farm Code
- Farm name and post code *(This information will be held in the strictest of confidence)*
- Date of visit
- Farm assurance scheme
- Flock production type
- Breed
- Flock size and numbers of houses in the farm
- Stocking density in kg/m²
- Placement date/s of chicks in the farm
- Feed information

About the house to be sampled

- House number
- Sex of the birds
- Number of birds per house

1. Bio-security at farm gate

- Any written policy available?
- Any barriers in place to prevent unauthorised entry on site?
- At gate, are there clear instructions for visitors how to proceed?
- Brief description of farm layout and movements' flow

- Is there equipment in place at farm gate to wash and disinfect vehicle wheels and arches? (If yes specified type of washing equipment)
- Is disinfectant being used? If so, is the concentration rate appropriate?
- Any verification system in place to verify the cleaning and disinfection procedures of the vehicles? Any record kept?
- After observation, were procedures in place and being observed by people?

2. <u>Visitors</u>

- Are records kept for site visitors (including previous sites visited)?
- Is access to visitors restricted to the minimum necessary?
- Is any bio-security information given to visitors before the visit?
- Is protective equipment supplied for visitors or own protective clothing could be used?
- At the site entrance, are facilities for hand cleansing, sanitisation and drying available for visitors?
- Is there any monitoring in place to verify the use of facilities by visitors?
- Were procedures being followed during the visit?

3. <u>Facilities & Protective clothing</u>

- Are hand-washing facilities available in suitable number and position? Are they available for each house?
- Are there enough footbaths, of adequate depth for covering all the foot part of the boot?
- Are there effective brushes or similar systems available for cleaning boots?
- Is disinfectant being used in the footbaths? If so, is it at the appropriate concentration rate?
- Are there any verification procedures to verify the disinfectant concentration?
- Are foot wear used by staff easy to clean and disinfect?
- Are facilities being effectively used by staff? Are there monitoring procedures in place to verify it?
- Is protective clothing changed and replaced when needed and at a set frequency?

- Is the correct protective clothing used by members of staff during the visit?

4. <u>Water Quality</u>

- Is water sanitizer used?
- Is any microbiological testing carried in the water system?

5. <u>Staff Awareness of Bio-security</u>

- Has training on bio-security been carried out for all staff? Are records kept?
- During the visit did staff appear to be taking a proactive approach to ensure site bio-security?

6. <u>Control of pests, wild birds & other livestock and waste control</u>

- Any pest control being carried out?
- Are other livestock or pets excluded from the site?
- Are there other livestock farms close to the site?
- Are fly screens available?
- How is waste disposed of?

7. <u>Catching</u>

- Is protective equipment supplied for catchers or their own protective clothing being used?
- Do catchers wear clean clothing and boots on each site?
- Are footbaths and hand wash facilities used by catchers? How are those practices being monitored by farm staff?
- Are breaks and changing facilities provided for catchers?

8. <u>Terminal Clean</u>

- Are written procedures for cleaning ad disinfection in place?
- Any verification procedures in place to verify the effectiveness of the terminal cleaning and disinfection operations?
- After how many days is new stock introduced?

APPENDIX 7

MLST PROFILE AMONG THE 141 CHICKEN ISOLATES

(UI unassigned isolates; novel STs are indicated in bold)

CCN	Production system	Origin	Campy ssp.	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST	Clonal complex
147	ST	Neck Skin	*C. jejuni*	8	17	5	2	10	59	6	ST 400	ST-353
181	ST	Neck Skin	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
197	ST	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
204	ST	Caeca	*C. jejuni*	87	124	30	82	113	44	74	ST 1761	UI
206	ST	Caeca	*C. jejuni*	8	10	2	2	11	12	6	ST 354	ST-354
211	ST	Caeca	*C. jejuni*	8	10	2	2	11	12	6	ST 354	ST-354
223	ST	Caeca	*C. jejuni*	87	124	30	82	113	44	74	ST 1761	UI
263	ST	Neck Skin	*C. jejuni*	76	22	165	98	146	254	16	ST 6557	UI
276	ST	Neck Skin	*C. jejuni*	76	22	165	98	146	254	16	ST 6557	UI
440	ST	Neck Skin	*C. jejuni*	8	10	2	2	11	12	6	ST 354	ST-354
457	ST	Neck Skin	*C. jejuni*	8	10	2	2	11	12	6	ST 354	ST-354
460	ST	Neck Skin	*C. jejuni*	8	10	2	2	11	12	6	ST 354	ST-354
486	ST	Caeca	*C. jejuni*	8	10	2	2	11	12	6	ST 354	ST-354
502	ST	Caeca	*C. coli*	33	39	30	82	104	56	17	ST 827	ST-828
504	ST	Caeca	*C. jejuni*	8	10	2	2	11	12	6	ST 354	ST-354
740	IW	Caeca	*C. jejuni*	9	2	4	62	4	5	12	ST 2030	ST-257
767	IW	Caeca	*C. jejuni*	7	17	5	62	1	67	6	ST 2976	ST-353
778	IW	Caeca	*C. jejuni*	9	2	4	62	4	5	12	ST 2030	ST-257

CCN	Production system	Origin	Campy ssp.	aspA	glnA	gltA	glyA	pgm	tkt	uncA	ST	Clonal complex
837	IW	Caeca	*C. coli*	33	39	30	82	104	43	17	ST 828	ST-828
840	IW	Caeca	*C. coli*	33	39	30	82	104	43	17	ST 828	ST-828
855	IW	Caeca	*C. coli*	33	39	30	82	104	43	17	ST 828	ST-828
857	IW	Caeca	*C. coli*	33	39	30	82	104	43	17	ST 828	ST-828
867	IW	Caeca	*C. jenuni*	2	7	10	4	189	7	1	ST 6568	ST-45
974	FI	Neck Skin	*C. jejuni*	7	2	5	2	10	3	6	ST 5	ST-353
975	FI	Neck Skin	*C. coli*	87	124	30	82	113	47	74	ST 4558	UI
1038	FI	Caeca	*C. coli*	87	124	30	82	113	47	74	ST 4558	UI
1042	FI	Caeca	*C. coli*	87	124	30	82	113	47	74	ST 4558	UI
1047	FI	Caeca	*C. coli*	87	124	30	82	113	47	74	ST 4558	UI
1064	FI	Caeca	*C. coli*	87	124	30	82	113	47	74	ST 4558	UI
1065	FI	Caeca	*C. coli*	87	124	30	82	113	7	74	ST 6569	UI
1066	FI	Caeca	*C. coli*	87	124	30	82	113	47	74	ST 4558	UI
1143	ST	Neck Skin	*C. jejuni*	4	7	10	4	42	7	1	ST 137	ST-45
1238	IW	Neck Skin	*C. jejuni*	7	17	5	62	1	67	6	ST 2976	ST-353
1256	IW	Caeca	*C. jejuni*	8	10	2	2	11	12	6	ST 354	ST-354
1284	IW	Caeca	*C. jejuni*	7	17	5	62	1	67	6	ST 2976	ST-353
1289	IW	Caeca	*C. jejuni*	22	15	4	64	74	25	23	ST 1709	ST-1034
1297	IW	Caeca	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1304	IW	Caeca	*C. jejuni*	7	17	2	15	23	3	12	ST 51	ST-443
1311	IW	Caeca	*C. jejuni*	2	75	4	48	141	34	1	ST 814	ST-661
1321	IW	Caeca	*C. jejuni*	2	75	4	48	141	34	1	ST 814	ST-661
1325	IW	Caeca	*C. jejuni*	7	2	5	2	10	3	6	ST 5	ST-353

CCN	Production system	Origin	Campy ssp.	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST	Clonal complex
1328	IW	Neck Skin	*C. jejuni*	2	75	4	48	141	34	1	ST 814	ST-661
1336	IW	Neck Skin	*C. jejuni*	2	75	4	48	141	34	1	ST 814	ST-661
1337	FR	Caeca	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1340	FR	Caeca	*C. jejuni*	4	22	10	4	1	7	1	ST 2197	ST-45
1351	FR	Caeca	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1358	FR	Caeca	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1361	FR	Neck Skin	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1371	FR	Neck Skin	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1386	FR	Caeca	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1389	FR	Caeca	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1390	FR	Caeca	*C. jejuni*	7	17	5	62	1	67	1	ST 2976	ST-353
1404	FR	Neck Skin	*C. jejuni*	4	7	10	4	42	7	1	ST 137	ST-45
1406	FR	Neck Skin	*C. jejuni*	4	7	10	4	1	7	6	ST 2223	ST 45
1412	FR	Neck Skin	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST 45
1416	ST	Neck Skin	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257
1418	ST	Neck Skin	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257
1419	ST	Caeca	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257
1420	ST	Caeca	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257
1421	IW	Neck Skin	*C. jejuni*	2	28	29	64	238	3	5	ST 2211	UI
1422	IW	Neck Skin	*C. jejuni*	9	2	4	62	4	5	12	ST 2030	ST-257
1424	IW	Caeca	*C. jejuni*	7	28	4	28	17	34	12	ST 573	ST-573
1429	IW	Neck Skin	*C. jejuni*	2	28	29	64	238	3	35	ST 2211	UI
1430	IW	Neck Skin	*C. jejuni*	7	2	5	2	10	67	6	ST 6560	ST-353

CCN	Production system	Origin	Campy ssp.	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST	Clonal complex
1432	IW	Neck Skin	*C. jejuni*	7	2	5	2	10	3	6	ST 5	ST-353
1434	ST	Caeca	*C. jejuni*	4	4	1	2	7	1	5	ST 739	ST-48
1436	ST	Caeca	*C. jejuni*	2	4	1	2	7	1	5	ST 48	ST-48
1437	FR	Neck Skin	*C. jejuni*	4	7	10	4	42	7	1	ST 137	ST-45
1442	FR	Caeca	*C. jejuni*	4	7	10	4	42	7	1	ST 137	ST-45
1443	FR	Caeca	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1445	FR	Caeca	*C. jejuni*	4	7	10	4	42	7	1	ST 137	ST-45
1449	FR	Neck Skin	*C. jejuni*	4	22	10	4	1	7	1	ST 2197	ST-45
1450	IW	Neck Skin	*C. jejuni*	8	1	6	3	2	1	12	ST 2135	ST-21
1451	IW	Neck Skin	*C. jejuni*	8	1	6	3	2	1	12	ST 2135	ST-21
1457	IW	Neck Skin	*C. jejuni*	2	28	29	64	238	3	35	ST 2211	UI
1460	IW	Caeca	*C. jejuni*	7	28	4	28	17	34	12	ST 573	ST-573
1462	FR	Neck Skin	*C. jejuni*	2	1	1	3	2	1	5	ST 21	ST-21
1465	FR	Neck Skin	*C. jejuni*	2	1	1	3	2	1	5	ST 21	ST-21
1472	FR	Caeca	*C. jejuni*	2	1	1	3	2	1	5	ST 21	ST-21
1478	FR	Neck Skin	*C. jejuni*	9	2	4	62	2	5	6	ST 6033	ST-257
1479	FR	Caeca	*C. jejuni*	2	1	1	3	2	1	5	ST 21	ST-21
1482	FR	Caeca	*C. jejuni*	2	1	1	3	2	1	5	ST 21	ST-21
1487 *	FR	Neck Skin	*C. jejuni*	2	1	1	3	2	1	5	ST 21	ST-21
1490 *	FR	Neck Skin	*C. jejuni*	2	1	1	3	2	1	5	ST 21	ST-21
1496	FR	Caeca	*C. jejuni*	2	1	1	3	2	1	5	ST 21	ST-21
1499	FR	Neck Skin	*C. jejuni*	7	17	2	15	23	3	12	ST 51	ST-443
1508	FR	Caeca	*C. jejuni*	7	17	2	15	23	3	12	ST 51	ST-443

CCN	Production system	Origin	Campy ssp.	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST	Clonal complex
1509	FR	Caeca	*C. jejuni*	4	7	10	4	1	7	1	ST 45	ST-45
1513	IW	Neck Skin	*C. jejuni*	24	2	2	2	10	3	3	ST 5136	ST-464
1516	IW	Caeca	*C. jejuni*	2	7	10	4	1	7	1	ST 233	ST-45
1518	IW	Caeca	*C. jejuni*	2	7	10	4	1	7	1	ST 233	ST-45
1519	IW	Caeca	*C. jejuni*	2	7	10	4	1	7	1	ST 233	ST-45
1521	IW	Caeca	*C. jejuni*	2	7	10	4	1	7	1	ST 233	ST-45
1522	IW	Caeca	*C. jejuni*	2	7	10	4	1	7	1	ST 233	ST-45
5014	ST	Breast Meat	*C. jejuni*	9	2	4	62	4	4	6	ST 3444	ST-257
5019	ST	Breast Meat	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257
5032	ST	Liver	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5039	ST	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5040	ST	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5041	ST	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5043	ST	Neck Skin	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5046	ST	Neck Skin	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5047	ST	Caeca	*C. coli*	33	39	30	391	104	47	17	ST 4422	ST 828
5049	ST	Caeca	*C. jejuni*	7	71	2	62	11	12	6	ST 1489	ST-354
5051	ST	Caeca	*C. coli*	33	39	30	391	104	47	17	ST 4422	ST 828
5052	ST	Caeca	*C. coli*	33	39	30	82	104	43	17	ST 828	ST 828
5053	ST	Breast Meat	*C. jejuni*	2	1	1	3	2	1	17	ST 797	ST-21
5055	ST	Neck Skin	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257
5056	ST	Neck Skin	*C. jejuni*	9	2	2	62	4	5	6	ST 257	ST-257
5057	ST	Neck Skin	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257

CCN	Production system	Origin	Campy ssp.	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST	Clonal complex
5062	ST	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5066	ST	Breast Meat	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257
5067	ST	Breast Meat	*C. jejuni*	9	2	4	62	4	5	6	ST 257	ST-257
5070	FI	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5072	FI	Neck Skin	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5074	FI	Neck Skin	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5075	FI	Neck Skin	*C. jejuni*	24*	2	2	2	10	3	1	ST 464	ST-464
5076	FI	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5077	FI	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5078	FI	Caeca	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5080	ST	Caeca	*C. jejuni*	7	28	4	28	23	3	12	ST 5148	ST-573
5092	FI	Breast Meat	*C. jejuni*	24	2	2	2	10	3	1	ST 464	ST-464
5100	FI	Breast Meat	*C. jejuni*	4	7	10	4	7	7	1	ST 1695	ST-45
5101	FI	Breast Meat	*C. jejuni*	7	4	2	2	19	1	6	ST 1900	ST-658
5104	FI	Breast Meat	*C. jejuni*	1	3	6	4	3	3	3	ST 22	ST-22
5121	FI	Caeca	*C. coli*	33	39	30	82	113	47	17	ST 825	ST-828
5124	FI	Caeca	*C. coli*	33	39	30	82	113	47	17	ST 825	ST-828
5125	FI	Caeca	*C. coli*	33	39	30	82	113	47	17	ST 825	ST-828
5126	IW	Caeca	*C. coli*	33	39	30	82	113	47	17	ST 825	ST-828
5130	FI	Neck Skin	*C. coli*	33	39	30	82	113	47	17	ST 825	ST-828
5132	FI	Neck Skin	*C. coli*	33	39	30	82	113	47	17	ST 825	ST-828
5134	FI	Breast Meat	*C. jejuni*	2	1	12	3	2	1	5	ST 50	ST-21
5136	FI	Breast Meat	*C. jejuni*	7	17	2	15	23	3	12	ST 51	ST-443

CCN	Production system	Origin	Campy ssp.	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST	Clonal complex
5163	FI	Caeca	*C. jejuni*	4	7	10	1	1	7	1	ST 25	ST-45
5173	ST	Neck Skin	*C. coli*	33	39	30	82	104	43	17	ST 828	ST-828
5176	ST	Neck Skin	*C. coli*	33	39	30	82	104	43	17	ST 828	ST-828
5177	ST	Caeca	*C. coli*	33	39	30	82	104	43	17	ST 828	ST-828
5179	ST	Caeca	*C. coli*	33	39	30	82	104	43	17	ST 828	ST-828
5190	ST	Breast Meat	*C. coli*	33	39	30	82	104	56	17	ST 827	ST-828
5193	ST	Breast Meat	*C. coli*	33	39	30	82	104	56	17	ST 827	ST-828
5214	ST	Breast Meat	*J. jeuni*	4	7	10	4	1	7	1	ST 45	ST-45

REFERENCES

Abulreesh, H. H., T. A. Paget, et al. (2005). "Recovery of thermophilic campylobacters from pond water and sediment and the problem of interference by background bacteria in enrichment culture." Water Research **39**: 2877-2882.

Achen, M., T. Y. Morishita, et al. (1998). "Shedding and colonization of Campylobacter jejuni in broilers from day-of-hatch to slaughter age." Avian Diseases **42**(4): 732-737.

Adak, G. K., S. M. Long, et al. (2002). "Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000." Gut **51**(6): 832-841.

Adak, G. K., S. M. Meakins, et al. (2005). "Disease risks from foods, England and Wales, 1996-2000." Emerging Infectious Diseases **11**(3): 365-372.

Adkin, A., E. Hartnett, et al. (2006). "Use of a systematic review to assist the development of Campylobacter control strategies in broilers." Journal of Applied Microbiology **100**(2): 306-315.

Advisory Committee on the Microbiological Safety of Food (2005). "Second Report on Campylobacter."

Aho, M., L. Nuotio, et al. (1992). "Competitive exclusion of campylobacters from poultry with K-bacteria and Broilact." International Journal of Food Microbiology **15**(3-4): 265-275.

Allain, V., L. Mirabito, et al. (2009). "Skin lesions in broiler chickens measured at the slaughterhouse: relationships between lesions and between their prevalence and rearing factors." British Poultry Science **50**(4): 407-417.

Allen, V. M., S. A. Bull, et al. (2007). "Campylobacter spp. contamination of chicken carcasses during processing in relation to flock colonisation." International Journal of Food Microbiology **113**(1): 54-61.

Allen, V. M. and D. G. Newell (2005). "Evidence for the effectiveness of biosecurity to exclude Campylobacter from poultry flocks." Food Standards Agency Report

Allen, V. M., A. M. Ridley, et al. (2011). "Influence of production system on the rate of onset of Campylobacter colonization in chicken flocks reared extensively in the United Kingdom." British Poultry Science **52**(1): 30-39.

Allos, B. (1997). "Association between Campylobacter infection and Guillain-Barré syndrome." J Infect Dis. **176**(2): 125-128.

Allos, B. M. (2001). "Campylobacter jejuni Infections: update on emerging issues and trends." Clinical Infectious Diseases **32**(8): 1201-1206.

Allos, B. M. and M. J. Blaser (1995). "Campylobacter jejuni and the expanding spectrum of related infections." Clinical Infectious Diseases **20**(5): 1092-1099; quiz 1100-1091.

Altekruse, S. F., N. J. Stern, et al. (1999). "Campylobacter jejuni--an emerging foodborne pathogen." Emerging Infectious Diseases **5**(1): 28-35.

Alter, T., F. Gaull, et al. (2005). "Distribution of Campylobacter jejuni strains at different stages of a turkey slaughter line." Food Microbiology **22**: 345-351.

Alter, T. and K. Scherer (2006). "Stress response of Campylobacter spp. and its role in food processing." Journal of Veterinary Medicine Series B **53**(8): 351-357.

Annan-Prah, A. and M. Janc (1988). "The mode of spread of Campylobacter jejuni/coli to broiler flocks." Zentralblatt Fuer Veterinaermedizin Reihe B **35**(1): 11-18.

Arnold, J. W. and S. Silvers (2000). "Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation." Poultry Science **79**(8): 1215-1221.

Arthur, J. and G. A. Albers (2003). "Industrial perpective on problema and issues associated with poultry breeding." Poultry Genetics, Breeding and Biotechnology.

Atanassova, V., F. Reich, et al. (2007). "Prevalence of Campylobacter spp. in turkey meat from a slaughterhouse and in turkey meat retail products." FEMS Immunology & Medical Microbiology **49**(1): 141-145.

Audran, X. (2010). "Poultry and Products Annual." GAIN Report

AVEC (2011). "Annual Report 2011."

Aviagen (2012). "Ross 308 Broiler."

Avrain, L., L. Allain, et al. (2003). "Disinfectant susceptibility testing of avian and swine Campylobacter isolates by a filtration method." Veterinary Microbiology **96**(1): 35-40.

Bahrndorff, S., L. Rangstrup-Christensen, et al. (2013). "Foodborne Disease Prevention and Broiler Chickens with Reduced Campylobacter Infection." Emerging Infectious Diseases **19**(3).

Bardoňa, B., M. Kolářb, et al. (2011). "Prevalence of thermotolerant Campylobacter spp. in broilers at retail in the Czech Republic and their antibiotic resistance." Food control **22**(2): 328-332.

Barrios, P. R., J. Reiersen, et al. (2006). "Risk factors for Campylobacter spp. colonization in broiler flocks in Iceland." Preventive Veterinary Medicine **74**(4): 264-278.

Bashor, M. P., P. A. Curtis, et al. (2004). "Effects of carcass washers on Campylobacter contamination in large broiler processing plants." Poultry Science **83**(7): 1232-1239.

Bashor, M. P., P. A. Curtis, et al. (2004). "Effects of carcass washers on Campylobacter contamination in large broiler processing plants." Poultry Science **83**(1232-1239).

Batz, M. B., M. P. Doyle, et al. (2005). "Attributing illness to food." Emerging Infectious Diseases **11**(7): 993-999.

Baze, W. B. and B. J. Bernacky (2002). "Campylobacter-induced fetal death in a rhesus monkey." Veterinary Pathology **39**(5): 605-607.

Beery, J. T., M. B. Hugdahl, et al. (1988). "Colonization of gastrointestinal tracts of chicks by Campylobacter jejuni." Applied & Environmental Microbiology **54**(10): 2365-2370.

Bennett, R., Kehlbacher, A., Balcombe, K. (2012). "A method for the economic valuation of animal welfare benefits using a single welfare score." Animal Welfare, 21 (S1). pp. 125-130 **21 (S1)**: 125-130.

Berndtson, E., M. L. Danielsson-Tham, et al. (1996). "Campylobacter incidence on a chicken farm and the spread of Campylobacter during the slaughter process." International Journal of Food Microbiology **32**(1-2): 35-47.

Berndtson, E., U. Emanuelsonb, et al. (1996). "A 1-year epidemiological study of campylobacters in 18 Swedish chicken farms." Preventive Veterinary Medicine **26**(3-4): 167-185.

Berndtson, E., M. Tivemo, et al. (1992). "Distribution and numbers of Campylobacter in newly slaughtered broiler chickens and hens." International Journal of Food Microbiology **15**(1-2): 45-50.

Berrang, M. E., R. J. Buhr, et al. (2001). "Broiler carcass contamination with Campylobacter from feces during defeathering." Journal of Food Protection **64**(12): 2063-2066.

Berrang, M. E. and J. A. Dickens (2000). "Presence and Level of Campylobacter spp. on Broiler Carcasses Throughout the Processing Plant." The Journal of Applied Poultry Research **9**(1): 43-47.

Berrang, M. E., R. J. Meinersmann, et al. (2008). "The effect of chilling in cold air or ice water on the microbiological quality of broiler carcasses and the population of Campylobacter." Poultry Science **87**(5): 992-998.

Berrang, M. E. and J. K. Northcutt (2005). "Use of Water Spray and Extended Drying Time to Lower Bacterial Numbers on Soiled Flooring from Broiler Transport Coops." Poultry Science **84**(1797-1801).

Berrang, M. E., J. K. Northcutt, et al. (2004). "Recovery of Campylobacter from broiler feces during extended storage of transport cages." Poultry Science **83**(7): 1213-1217.

Berrang, M. E., W. R. Windham, et al. (2011). "Campylobacter, Salmonella, and Escherichia coli on broiler carcasses subjected to a high pH scald and low pH postpick chlorine dip." Poultry Science **90**(4): 896-900.

Bessei, W. (2006). "Welfare of broilers: a review." World's Poultry Science Journal, **62**.

Beuchat, L. R. (1986). "Methods for detecting and enumerating Campylobacter jejuni and Campylobacter coli in poultry." Poultry Science **65**(12): 2192-2198.

Bilgili, S. F., M. A. Alley, et al. (2006). "Influence of Age and Sex on Footpad Quality and Yield in Broiler Chickens Reared on Low and High Density Diets." The Journal of Applied Poultry Research **15** (3): 433-441.

Birkhead, G., R. L. Vogt, et al. (1988). "A multiple-strain outbreak of Campylobacter enteritis due to consumption of inadequately pasteurized milk." Journal of Infectious Diseases **157**(5): 1095-1097.

Black, R., M. Levine, et al. (1988). "Experimental Campylobacter jejuni Infection in Humans." The journal of infectious diseases **157**.

Blankenship, L. C. and S. E. Craven (1982). "Campylobacter jejuni survival in chicken meat as a function of temperature " Applied and Environmental Microbiology 1982 July; 44(1): 88–92. **44**(1): 88-92.

Blaser, M. J. (1997). "Epidemiologic and clinical features of Campylobacter jejuni infections." Journal of Infectious Diseases **176 Suppl 2**: S103-105.

Bodhidatta, L., N. Vithayasai, et al. (2002). "Bacterial enteric pathogens in children with acute dysentery in Thailand: increasing importance of quinolone-resistant Campylobacter." Southeast Asian Journal of Tropical Medicine & Public Health **33**(4): 752-757.

Bogaardt, M. J., M. Mangen, et al. (2004). "Controlling Campylobacter in the chicken meat chain.Towards a decision support model." RIVM report 250911005/2004.

Bokkers, E. and P. Koene (2003). "Behaviour of fast- and slow growing broilers to 12 weeks of age and the physical consequences." Applied Animal Behaviour Science **81**(1): 57-72.

Bolton, F. J. and D. Coates (1983). "A study of the oxygen and carbon dioxide requirements of thermophilic campylobacters." Journal of Clinical Pathology **36**(7): 829-834.

Bouwknegt, M., A. W. van de Giessen, et al. (2004). "Risk factors for the presence of Campylobacter spp. in Dutch broiler flocks." Preventive Veterinary Medicine **62**(1): 35-49.

Boysen, L. and H. Rosenquist (2009). "Reduction of thermotolerant Campylobacter species on broiler carcasses following physical decontamination at slaughter." Journal of Food Protection **72**(3): 497-502.

Bull, S. A., V. M. Allen, et al. (2006). "Sources of Campylobacter spp. colonizing housed broiler flocks during rearing." Applied & Environmental Microbiology **72**(1): 645-652.

Bull, S. A., A. Thomas, et al. (2008). "Flock health indicators and Campylobacter spp. in commercial housed broilers reared in Great Britain." Applied & Environmental Microbiology **74**(17): 5408-5413.

Burkholder, K. M., K. L. Thompson, et al. (2008). "Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella enteritidis colonization in broilers." Poultry Science **87**(9): 1734-1741.

Byrd, J. A., D. E. Corrier, et al. (1998). "Effect of feed withdrawal on Campylobacter in the crops of market-age broiler chickens." Avian Diseases **42**(4): 802-806.

Byrne, C. M., M. Clyne, et al. (2007). "Campylobacter jejuni adhere to and invade chicken intestinal epithelial cells in vitro." Microbiology & Immunology **153**: 561–569.

Callaway, T., J. Morrow, et al. (2006). "Social stress increases fecal shedding of Salmonella typhimurium by early weaned piglets." Current issues in intestinal microbiology **7**(2): 65-71.

Callicott, K. A., V. Friethriksdottir, et al. (2006). "Lack of evidence for vertical transmission of Campylobacter spp. in chickens." Applied & Environmental Microbiology **72**(9): 5794-5798.

Callicott, K. A., H. Hargardottir, et al. (2008). "Broiler Campylobacter contamination and human campylobacteriosis in Iceland." Applied & Environmental Microbiology **74**(21): 6483-6494.

Carlone, G. and J. Lascelles (1982). "Aerobic and anaerobic respiratory systems in Campylobacter fetus subsp. jejuni grown in atmospheres containing hydrogen." J Bacteriol. **152 (1)**: 306-314.

Carrillo, C. L., R. J. Atterbury, et al. (2005). "Bacteriophage Therapy To Reduce Campylobacter jejuni Colonization of Broiler Chickens." Applied and Enviroromental Microbilogy **71**(11): 6554-6563.

Carvalho, A. C., G. M. Ruiz-Palacios, et al. (2001). "Molecular characterization of invasive and noninvasive Campylobacter jejuni and Campylobacter coli isolates." Journal of Clinical Microbiology **39**(4): 1353-1359.

Cawthraw, S. A., T. M. Wassenaar, et al. (1996). "Increased colonization potential of Campylobacter jejuni strain 81116 after passage through chickens and its implication on the rate of transmission within flocks." Epidemiology & Infection **117**(1): 213-215.

Chantarapanont, W., M. Berrang, et al. (2003). "Direct microscopic observation and viability determination of Campylobacter jejuni on chicken skin." Journal of Food Protection **66**(12): 2222-2230.

Chaveerach, P., D. A. Keuzenkamp, et al. (2002). "In vitro study on the effect of organic acids on Campylobacter jejuni/coli populations in mixtures of water and feed." Poultry Science **81**(621-628).

Chen, H. C. and N. J. Stern (2001). "Competitive exclusion of heterologous Campylobacter spp. in chicks." Applied & Environmental Microbiology **67**(2): 848-851.

Chmielewski, R. and J. F. Frank (2003). "Biofilm formation and control in food processing facilities " Comprehensive Reviews in Food Science and Food Safety **2**(22-32).

Chuma, T., S. Hashimoto, et al. (2000). "Detection of thermophilic Campylobacter from sparrows by multiplex PCR: the role of sparrows as a source of contamination of broilers with Campylobacter." Journal of Veterinary Medical Science **62**(12): 1291-1295.

Chynoweth, R. W., J. A. Hudson, et al. (1998). "Aerobic growth and survival of Campylobacter jejuni in food and stream water." Letters in Applied Microbiology **27**(6): 341-344.

Cody, A. J., L. Clarke, et al. (2010). "Ciprofloxacin-resistant campylobacteriosis in the UK." Lancet **376**(9757): 1987.

Cogan, T. A., S. F. Bloomfield, et al. (1999). "The effectiveness of hygiene procedures for prevention of cross-contamination from chicken carcases in the domestic kitchen." Letters in Applied Microbiology **29**(5): 354-358.

Cogan, T. A. and T. J. Humphrey (2003). "The rise and fall of Salmonella Enteritidis in the UK." Journal of Applied Microbiology **94 Suppl**: 114S-119S.

Cogan, T. A., J. Slader, et al. (2002). "Achieving hygiene in the domestic kitchen: the effectiveness of commonly used cleaning procedures." Journal of Applied Microbiology **92**(5): 885-892.

Cogan, T. A., A. O. Thomas, et al. (2007). "Norepinephrine increases the pathogenic potential of Campylobacter jejuni." Gut **56**(8): 1060-1065.

Coker, A. O. and A. O. Adefeso (1994). "The changing patterns of Campylobacter jejuni/coli in Lagos, Nigeria after ten years." East African Medical Journal **71**(7): 437-440.

Coker, A. O., R. D. Isokpehi, et al. (2002). "Human campylobacteriosis in developing countries." Emerging Infectious Diseases **8**(3): 237-244.

Cole, K., A. M. Donoghue, et al. (2004). "Isolation and prevalence of Campylobacter in the reproductive tracts and semen of commercial turkeys." Avian Diseases **48**(3): 625-630.

Colles, F. M., K. Jones, et al. (2003). "Genetic diversity of Campylobacter jejuni isolates from farm animals and the farm environment." Applied & Environmental Microbiology **69**(12): 7409-7413.

Colles, F. M., T. A. Jones, et al. (2008). "Campylobacter infection of broiler chickens in a free-range environment." Environmental Microbiology **10**(8): 2042-2050.

Colles, F. M. and M. C. Maiden (2012). "Campylobacter sequence typing databases: applications and future prospects." Microbiology **158**: 2695-2709.

Colles, F. M. and M. C. J. Maiden (2012). "Campylobacter sequence typing databases: applications and future prospects." Microbiology **158**(Pt 11): 2695-2709.

Colles, F. M., N. D. McCarthy, et al. (2011). "The prevalence of Campylobacter amongst a free-range broiler breeder flock was primarily affected by flock age." PLoS ONE [Electronic Resource] **6**(12): e22825.

Colles, F. M., N. D. McCarthy, et al. (2010). "Comparison of Campylobacter populations isolated from a free-range broiler flock before and after slaughter." International Journal of Food Microbiology **137**(2-3): 259-264.

Conlan, A. J. K., C. Coward, et al. (2007). "Campylobacter jejuni colonization and transmission in broiler chickens: a modelling perspective." Journal of the Royal Society Interface **4**(16): 819-829.

Cools, I., M. Uyttendaele, et al. (2005). "Persistence of Campylobacter jejuni on surfaces in a processing environment and on cutting boards." Letters in Applied Microbiology **40**(6): 418-423.

Corry, J. E. and H. I. Atabay (2001). "Poultry as a source of Campylobacter and related organisms." Symposium Series/The Society for Applied Microbiology(30): 96S-114S.

Corry, J. E., D. E. Post, et al. (1995). "Culture media for the isolation of campylobacters." International Journal of Food Microbiology **26**(1): 43-76.

Cosansu, S. and K. Ayhan (2010). "Effect of lactic and acetic acid treatments on Campylobacter Jejuni inoculated onto chicken leg and breast meat during storage at 4C and -18." Journal of Food Processing and Preservation **34**(1): 98-113.

Cox, N. A., C. L. Hofacre, et al. (2005). "Presence of Campylobacter jejuni in various organs one hour, one day, and one week following oral or intracloacal inoculations of broiler chicks." Avian Diseases **49**(1): 155-158.

Cox, N. A., L. J. Richardson, et al. (2006). "Natural presence of Campylobacter spp. in various internal organs of commercial broiler breeder hens." Avian Diseases **50**(3): 450-453.

Crane, R., R. Davenport, et al. (2012). "Farm Business Survey 2010/11 Poultry Production in England."

Craven, S. E., N. J. Stern, et al. (2000). "Determination of the incidence of Salmonella spp., Campylobacter jejuni and Clostridium perfringens in wild birds near broiler chicken houses by sampling intestinal droppings." Avian Diseases **44**(3): 715-720.

Davis, M. A. and D. E. Conner (2007). "Survival of Campylobacter jejuni on poultry skin and meat at varying temperatures." Poultry Science **86**(4): 765-767.

Dawkins, M. S., C. A. Donnelly, et al. (2004). "Chicken welfare is influenced more by housing conditions than by stocking density." Nature **427**(6972): 342-344.

de Haan, C. P., R. Kivisto, et al. (2010). "Decreasing trend of overlapping multilocus sequence types between human and chicken Campylobacter jejuni isolates over a decade in Finland." Applied & Environmental Microbiology **76**(15): 5228-5236.

de Haan, C. P. A., R. Kivisto, et al. (2010). "Decreasing trend of overlapping multilocus sequence types between human and chicken Campylobacter jejuni isolates over a decade in Finland." Applied & Environmental Microbiology **76**(15): 5228-5236.

de Jong, A., E. van Asselt, et al. (2012). "Extreme Heat Resistance of Food Borne Pathogens Campylobacter jejuni, Escherichia coli, and Salmonella typhimurium on Chicken Breast Fillet during Cooking." International Journal of Microbiology **2012**.

de Jong, A. E., L. Verhoeff-Bakkenes, et al. (2008). "Cross-contamination in the kitchen: effect of hygiene measures." Journal of Applied Microbiology **105**(2): 615-624.

de Jong, I., C. Berg, et al. (2012). "Scientific report updating the EFSA opinions on the welfare of broilers and broiler breeders."

de Zoete, M. R., J. P. M. van Putten, et al. (2007). "Vaccination of chickens against Campylobacter." Vaccine **25**(30): 5548-5557.

DEFRA (2006). "The structure of the United Kingdom poultry industry: Commercial poultry sector ".

DEFRA (2009). "Guide to the National Control Programme for Salmonella in broiler flocks."

DEFRA (2011). "Update on the Salmonella national control programmes in poultry."

DEFRA (2013). "Poultry and poultry meat statistics."

Denton, K. J. and T. Clarke (1992). "Role of Campylobacter jejuni as a placental pathogen." Journal of Clinical Pathology **45**(2): 171-172.

Dhillon, A. S., H. L. Shivaprasad, et al. (2006). "Campylobacter jejuni infection in broiler chickens." Avian Diseases **50**(1): 55-58.

Dincer, A. H. and T. Baysal (2004). "Decontamination techniques of pathogen bacteria in meat and poultry." Critical Reviews in Microbiology **30**(3): 197-204.

Dingle, K. E., F. M. Colles, et al. (2002). "Molecular characterization of Campylobacter jejuni clones: a basis for epidemiologic investigation." Emerging Infectious Diseases **8**(9): 949-955.

Dingle, K. E., F. M. Colles, et al. (2001). "Multilocus sequence typing system for Campylobacter jejuni." Journal of Clinical Microbiology **39**(1): 14-23.

Dingle, K. E., N. D. McCarthy, et al. (2008). "Extended sequence typing of Campylobacter spp., United Kingdom." Emerging Infectious Diseases **14**(10): 1620-1622.

Dingle, K. E., N. Van Den Braak, et al. (2001). "Sequence typing confirms that Campylobacter jejuni strains associated with Guillain-Barré and Miller-Fisher syndromes are of diverse genetic lineage, serotype, and flagella type." Journal of Clinical Microbiology **39(9):3346-9**(9): 3346-3349.

Dohmsa, J. and A. Metzb (1991). "Stress — mechanisms of immunosuppression." Veterinary Immunology and Immunopathology **30**(1): 89-109.

EFSA (2008). "Assessing health benefits of controlling Campylobacter in the food chain."

EFSA (2010a). "Analysis of the baseline survey on the prevalence of Campylobacter in broiler batches and of Campylobacter and Salmonella on broiler carcasses in the EU,2008 Part A: Campylobacter and Salmonella prevalence estimates." EFSA Journal 2010 **8**(3): 1503.

EFSA (2010b). "Analysis of the baseline survey on the prevalence of Campylobacter in broiler batches and of Campylobacter and Salmonella on broiler carcasses in the EU,2008 Part B: Analysis of factors associated with Campylobacter colonization of broiler batches and with Campylobacter contamination of broiler carcasses;and investigation of the culture method diagnostic characteristics used to analyse broiler carcass sample." EFSA **8**: 1522.

EFSA (2010c). "Scientific Opinion on the influence of genetic parameters on the welfare and the resistance to stress of commercial broilers." EFSA Journal **8**(7).

EFSA (2010d). "Scientific Opinion on Quantification of the risk posed by broiler meat to human campylobacteriosis in the EU." EFSA Journal **8**(1).

EFSA (2010e). "Special Eurobaromether 354, Food-related risks."

EFSA (2011). "Scientific Opinion on Campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain." EFSA Journal **9**(4).

EFSA (2011). "Scientific Opinion on Campylobacter in broiler meat production: control

options and performance objectives and/or targets." EFSA Journal 2011; 9(4):2105.

EFSA (2012). "The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2010." EFSA Journal **10(3):2597**(10(3)): 2597.

EFSA (2013). "Campylobacter."

EFSA (2013). "The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011." EFSA Journal **11**(4).

Ekdahl, K., B. Normann, et al. (2005). "Could flies explain the elusive epidemiology of campylobacteriosis?" BMC Infectious Diseases **5**: 11.

Ekstrand, C., B. Algers, et al. (1997). "Rearing conditions and foot-pad dermatitis in Swedish broiler chickens." Preventive Veterinary Medicine **31**(3-4): 167-174.

El-Shibiny, A., P. L. Connerton, et al. (2005). "Enumeration and diversity of campylobacters and bacteriophages isolated during the rearing cycles of free-range and organic chickens." Applied & Environmental Microbiology **71**(3): 1259-1266.

El-Shibiny, A., P. L. Connerton, et al. (2007). "Campylobacter succession in broiler chickens." Veterinary Microbiology **125**(3-4): 323-332.

Ellerbroek, L. I., J. Lienau, et al. (2010). "Campylobacter spp. in Broiler Flocks at Farm Level and the Potential for Cross-Contamination During Slaughter." Zoonoses and Public Health **57**(7-8): 81-88.

Elvers, K. T., V. K. Morris, et al. (2011). "Molecular Tracking, through Processing, of Campylobacter Strains Colonizing Broiler Flocks." Applied and Environmental Microbiology **77**(16): 5722-5729.

Engvall, A. (2001). "May organically farmed animals pose a risk for Campylobacter infections in humans?" Acta Veterinaria Scandinavica - Supplement **95**: 85-87.

Estevez, I. (2007). "Density allowances for broilers: where to set the limits?" Poultry Science **86**(6): 1265-1272.

Eurogroup for animals (2010). " Analysis of animal welfare issues in the European Union ".

Evans, S. J. and A. R. Sayers (2000). "A longitudinal study of campylobacter infection of broiler flocks in Great Britain." Preventive Veterinary Medicine **46**(3): 209-223.

Everest, P. (2007). "Stress and bacteria: microbial endocrinology." Gut **56**(8): 1037-1038.

FAO and WHO (2002). "Risk assessment of Campylobacter spp. in broiler chickens and Vibrio spp. in seafood."

FAO and WHO (2008). "Benefits and Risks of the Use of Chlorine-containing Disinfectants in Food Production and Food Processing." Report of a Joint FAO/WHO Expert Meeting.

Figueroa, G., M. Troncoso, et al. (2009). "Occurrence and enumeration of Campylobacter spp. during the processing of Chilean broilers." BMC Microbiology **9**: 94.

Finch, M. J. and P. A. Blake (1985). "Foodborne outbreaks of campylobacteriosis: the United States experience, 1980-1982." American Journal of Epidemiology **122**(2): 262-268.

Foley, S. L., A. M. Lynne, et al. (2009). "Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens." Infection, Genetics & Evolution **9**(4): 430-440.

FSA (2005). "Evidence for the effectiveness of biosecurity to exclude Campylobacter from poultry flocks."

FSA (2009). "UK-wide Survey of Salmonella and Campylobacter Contamination of Fresh and Frozen Chicken on Retail Sale."

FSA (2010). "The joint Goverment and Industry target to reduce Campylobacter in UK produced chickens by 2015."

FSA (2013). "A refreshed strategy to reduce campylobacteriosis from poultry ".

Gardiner, E. E., J. R. Hunt, et al. (1988). "Relationships between age, body weight, and season of the year and the incidence of sudden death syndrome in male broiler chickens." Poultry Science **67**(9): 1243-1249.

Georgsson, F., A. E. Thornorkelsson, et al. (2006). "The influence of freezing and duration of storage on Campylobacter and indicator bacteria in broiler carcasses." Food Microbiology **23**(7): 677-683.

Ghafir, Y., B. China, et al. (2007). "A seven-year survey of Campylobacter contamination in meat at different production stages in Belgium." International Journal of Food Microbiology **116**(1): 111-120.

Gillespie, I. A., S. J. O'Brien, et al. (2009). "Age patterns of persons with campylobacteriosis, England and Wales, 1990-2007." Emerging Infectious Diseases **15**(12): 2046-2048.

Gillespie, I. A., S. J. O'Brien, et al. (2002). "A case-case comparison of Campylobacter coli and Campylobacter jejuni infection: a tool for generating hypotheses." Emerging Infectious Diseases **8**(9): 937-942.

Gillespie, I. A., S. J. O'Brien, et al. (2008). "Demographic determinants for Campylobacter infection in England and Wales: implications for future epidemiological studies." Epidemiology & Infection **136**(12): 1717-1725.

Godley, A. (2007). "The Chicken, the Factory Farm and the Supermarket: the Emergence of the Modern Poultry Industry in Britain."

Godley, A. (2009). "Democratizing Luxury and the Contentious 'Invention of the Technological Chicken' in Britain." Business History Review **83**(2).

Godley, A. and B. Williams (2007). "The chicken, the factory farm and the supermarket: the emergence of the modern poultry industry in Britain."

Gonzalez, I., T. Garcia, et al. (2000). "Development of a combined PCR-culture technique for the rapid detection of Arcobacter spp. in chicken meat." Letters in Applied Microbiology **30**(3): 207-212.

Gormley, F. J., C. L. Little, et al. (2011). "A 17-year review of foodborne outbreaks: describing the continuing decline in England and Wales (1992-2008)." Epidemiology & Infection **139**(5): 688-699.

Gormley, F. J., M. Macrae, et al. (2008). "Has retail chicken played a role in the decline of human campylobacteriosis?" Applied & Environmental Microbiology **74**(2): 383-390.

Griekspoor, P., E. O. Engvall, et al. (2010). "Multilocus sequence typing of Campylobacter jejuni from broilers." Veterinary Microbiology **140**(1-2): 180-185.

Guerin, M. T., W. Martin, et al. (2007). "House-level risk factors associated with the colonization of broiler flocks with Campylobacter spp. in Iceland, 2001 - 2004." BMC Veterinary Research [Electronic Resource] **3**: 30.

Guerin, M. T., C. Sir, et al. (2010). "The change in prevalence of Campylobacter on chicken carcasses during processing: a systematic review." Poultry Science **89**(5): 1070-1084.

Gurgan, T. and K. S. Diker (1994). "Abortion associated with Campylobacter upsaliensis." Journal of Clinical Microbiology **32**(12): 3093-3094.

Habib, I., I. Sampers, et al. (2008). "Baseline data from a Belgium-wide survey of Campylobacter species contamination in chicken meat preparations and considerations for a reliable monitoring program." Applied & Environmental Microbiology **74**(17): 5483-5489.

Hakkinen, M. and C. Schneitz (1999). "Efficacy of a commercial competitive exclusion product against Campylobacter jejuni." British Poultry Science **40**(5): 619-621.

Hald, B., E. Rattenborg, et al. (2001). "Role of batch depletion of broiler houses on the occurrence of Campylobacter spp. in chicken flocks." Letters in Applied Microbiology **32**(4): 253-256.

Hald, B., H. Skovgard, et al. (2004). "Flies and Campylobacter infection of broiler flocks." Emerging Infectious Diseases **10**(8): 1490-1492.

Hald, B., A. Wedderkopp, et al. (2000). "Thermophilic Campylobacter spp. in Danish broiler production: A cross-sectional survey and a retrospective analysis of risk factors for occurrence in broiler flocks." Avian Pathology **29**: 123-131.

Hanning, I., R. Jarquin, et al. (2008). "Campylobacter jejuni as a secondary colonizer of poultry biofilms." Journal of Applied Microbiology **105**(4): 1199-1208.

Hannu, T., L. Mattila, et al. (2002). "Campylobacter-triggered reactive arthritis: a population-based study." Rheumatology **41**(3): 312-318.

Hansson, I., M. Ederoth, et al. (2005). "Transmission of Campylobacter spp. to chickens during transport to slaughter." Journal of Applied Microbiology **99**(5): 1149-1157.

Hansson, I., E. O. Engvall, et al. (2010). "Risk factors associated with the presence of Campylobacter-positive broiler flocks in Sweden." Preventive Veterinary Medicine **96**(1-2): 114-121.

Hansson, I., L. P. Forshell, et al. (2007). "Summary of the Swedish Campylobacter program in broilers, 2001 through 2005." Journal of Food Protection **70**(9): 2008-2014.

Hansson, I., N. Pudas, et al. (2010). "Within-flock variations of Campylobacter loads in caeca and on carcasses from broilers." International Journal of Food Microbiology **141**(1-2): 51-55.

Harms, R. H., B. L. Damron, et al. (1977). "Effect of wet litter and supplemental biotin and/or whey on the production of foot pad dermatitis in broilers." Poultry Science **56**(1): 291-296.

Harris, N. V., N. S. Weiss, et al. (1986). "The role of poultry and meats in the etiology of Campylobacter jejuni/coli enteritis." American Journal of Public Health **76**(4): 407-411.

Haughton, P. N., J. G. Lyng, et al. (2011). "Efficacy of high-intensity pulsed light for the microbiological decontamination of chicken, associated packaging, and contact surfaces." Foodborne Pathogens and Disease **8**(1): 109-117.

Havelaar, A. H., M.-J. J. Mangen, et al. (2007). "Effectiveness and efficiency of controlling Campylobacter on broiler chicken meat." Risk Analysis **27**(4): 831-844.

Havenstein, G. B., P. R. Ferket, et al. (2003). "Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets." Poultry Science **82**(10): 1500-1508.

Havenstein, G. B., P. R. Ferket, et al. (1994). "Growth, Livability, and Feed Conversion of 1957 vs 1991 Broilers When Fed “Typical” 1957 and 1991 Broiler Diets." Poultry Science **73**(12): 1785-1794.

Hayama, Y., T. Yamamoto, et al. (2011). "Simulation model for Campylobacter cross-contamination during poultry processing at slaughterhouses." Zoonoses & Public Health **58**(6): 399-406.

Hayes, R. J. and S. Bennett (1999). "Simple sample size calculation for cluster-randomized trials." International Journal of Epidemiology **28**(2): 319-326.

Hazeleger, W. C., J. A. Wouters, et al. (1998). "Physiological activity of Campylobacter jejuni far below the minimal growth temperature." Applied & Environmental Microbiology **64**(10): 3917-3922.

Health Protection Agency, H. (2011). "Over 90 per cent of campylobacter outbreaks at catering venues in 2011 linked to undercooked chicken liver pate."

Hedstrom, O. R., R. J. Sonn, et al. (1987). "Pathology of Campylobacter jejuni abortion in sheep." Veterinary Pathology **24**(5): 419-426.

Herman, L., M. Heyndrickx, et al. (2003). "Routes for Campylobacter contamination of poultry meat: epidemiological study from hatchery to slaughterhouse." Epidemiology & Infection **131**(3): 1169-1180.

Heuer, O. E., K. Pedersen, et al. (2001). "Prevalence and antimicrobial susceptibility of thermophilic Campylobacter in organic and conventional broiler flocks." Letters in Applied Microbiology **33**(4): 269-274.

Hillers, V. N., L. Medeiros, et al. (2003). "Consumer food-handling behaviors associated with prevention of 13 foodborne illnesses." Journal of Food Protection **66**(10): 1893-1899.

Hopkins, R. S. and A. S. Scott (1983). "Handling raw chicken as a source for sporadic Campylobacter jejuni infections." Journal of Infectious Diseases **148**(4): 770.

HPA (2011). "UK Standards for Microbiology Investigations, Identification of Campylobacter species." Bacteriology -- Identification **ID 23**(2.1).

HPA (2012). "Laboratory reports of Campylobacter sp infection in England and Wales reported to the Health Protection Agency 2000-2011."

Hubbard (2012). "Hubbard."

Hudson, W. R. and G. C. Mead (1987). "Factors affecting the survival of Campylobacter jejuni in relation to immersion scalding of poultry." Veterinary Record **121**(10): 225-227.

Hue, O., V. Allain, et al. (2011). "Campylobacter contamination of broiler caeca and carcasses at the slaughterhouse and correlation with Salmonella contamination." Food Microbiology **28**(5): 862-868.

Hue, O., S. Le Bouquin, et al. (2010). "Prevalence of and risk factors for Campylobacter spp. contamination of broiler chicken carcasses at the slaughterhouse." Food Microbiology **27**(8): 992-999.

Hugas, M., T. Eirini, et al. (2007). "Pros and cons of carcass decontamination: The role of the European Food Safety Authority " Meat Science.

Hum, S., M. Hornitzky, et al. (2009). "Ovine Campylobacteriosis."

Humphrey, J. T. (2013). "Zoonotic diseases, human health and farm animal welfare."

Humphrey, J. T. (2013). "Zoonotic diseases, human health and farm animal welfare: Campylobacter."

Humphrey, J. T. and D. G. Lanning (1987). "Salmonella and campylobacter contamination of broiler chicken carcasses and scald tank water: the influence of water pH." Journal Applied Bacteriology **63**: 21-25.

Humphrey, T. (2006). "Are happy chickens safer chickens? Poultry welfare and disease susceptibility." British Poultry Science **47**(4): 379—391.

Humphrey, T., S. J. O'Brien, et al. (2007). "Campylobacters as zoonotic pathogens: a food production perspective." International Journal of Food Microbiology **117**(3): 237-257.

Humphrey, T. J., A. Henley, et al. (1993). "The colonization of broiler chickens with Campylobacter jejuni: some epidemiological investigations." Epidemiology & Infection **110**(3): 601-607.

Hunter, S. M., M. E. Berrang, et al. (2009). "Genetic diversity of Campylobacter on broiler carcasses collected preevisceration and postchill in 17 U.S. poultry processing plants." Journal of Food Protection **72**(1): 49-54.

International Consultative Group on Food Irradiation (2009). "Safety of poultry meat: from farm to table."

ISO, I. O. f. S. (2006). "Microbiology of food and animal feeding stuff - Horizontal method for detection and enumeration of Campylobacter spp.-Part 2: Colony-count technique " DD ISO/TS 10272-2:2006.

Izat, A. L., F. A. Gardner, et al. (1988). "Incidence and level of Campylobacter jejuni in broiler processing." Poultry Science **67**(11): 1568-1572.

Jacobs-Reitsma, W. F. (1995). "Campylobacter bacteria in breeder flocks." Avian Diseases **39**(2): 355-359.

Jacobs-Reitsma, W. F., A. W. van de Giessen, et al. (1995). "Epidemiology of Campylobacter spp. at two Dutch broiler farms." Epidemiology & Infection **114**(3): 413-421.

James, C., C. Vincent, et al. (2006). "The primary chilling of poultry carcasses—a review." International Journal of Refrigeration **29**(6): 847-862.

Jeffrey, J. S., K. H. Tonooka, et al. (2001). "Prevalence of campylobacter spp. from skin, crop, and intestine of commercial broiler chicken carcasses at processing." Poultry Science **80**(9): 1390-1392.

Jennings, J. L., L. C. Sait, et al. (2011). "Campylobacter jejuni is associated with, but not sufficient to cause vibrionic hepatitis in chickens." Veterinary Microbiology **149**(1-2): 193-199.

Johannessen, G. S., G. Johnsen, et al. (2007). "Enumeration of thermotolerant Campylobacter spp. from poultry carcasses at the end of the slaughter-line." Letters in Applied Microbiology **44**(1): 92-97.

John Tang Yew Huat, S. A. A., Jalila Abu, Farinazleen Mohamad Ghazali, Tuan Zainazor Tuan Chilek, Noorlis Ahmad, Afriani Sandra, Mitsuaki Nishibuchi and Son Radu (2010). "Thermophilic Campylobacter spp. Occurrence on Chickens at Farm, Slaughter House and Retail." International Journal of Poultry Science **9** (2): 134-138.

Johnsen, G., H. Kruse, et al. (2006). "Genetic diversity and description of transmission routes for Campylobacter on broiler farms by amplified-fragment length polymorphism." Journal of Applied Microbiology **101**(5): 1130-1139.

Johnsen, G., H. Kruse, et al. (2007). "Genotyping of thermotolerant Campylobacter from poultry slaughterhouse by amplified fragment length polymorphism." Journal of Applied Microbiology **103**(2): 271-279.

Jones, F. S. and R. B. Little (1931). "The etiology of infectious diarrhea (winter scours) in cattle." J Exp Med **53(6)**: 835–843.

Jones, F. S., M. Orcutt, et al. (1931). "Vibrios (vibrio jejuni, n.sp.) associated with intestinal disorders of cows and calves." J Exp Med **53(6)**: 853–863.

Jore, S., H. Viljugrein, et al. (2010). "Trends in Campylobacter incidence in broilers and humans in six European countries, 1997-2007." Preventive Veterinary Medicine **93**(1): 33-41.

Jorgensen, F., R. Bailey, et al. (2002). "Prevalence and numbers of Salmonella and Campylobacter spp. on raw, whole chickens in relation to sampling methods." International Journal of Food Microbiology **76**(1-2): 151-164.

Jorgensen, F., J. Ellis-Iversen, et al. (2011). "Influence of season and geography on Campylobacter jejuni and C. coli subtypes in housed broiler flocks reared in Great Britain." Applied & Environmental Microbiology **77**(11): 3741-3748.

Jozwiak, A., O. Reichart, et al. "The occurrence of Campylobacter species in Hungarian broiler chickens from farm to slaughter." Journal of Veterinary Medicine Series B **53**(6): 291-294.

Julian, R. J. (1998). "Rapid growth problems: ascites and skeletal deformities in broilers." Poultry Science **77**(12): 1773-1780.

Kalischuk, L. D. and A. G. Buret (2010). "A role for Campylobacter jejuni-induced enteritis in inflammatory bowel disease?" American Journal of Physiology - Gastrointestinal & Liver Physiology **298**(1): G1-9.

Kapperud, G., E. Skjerve, et al. (1993). "Epidemiological investigation of risk factors for campylobacter colonization in Norwegian broiler flocks." Epidemiology & Infection **111**(2): 245-255.

Karenlampi, R., H. Rautelin, et al. (2007). "Longitudinal study of Finnish Campylobacter jejuni and C. coli isolates from humans, using multilocus sequence typing, including comparison with epidemiological data and isolates from poultry and cattle." Applied & Environmental Microbiology **73**(1): 148-155.

Katzav, M., P. Isohanni, et al. (2008). "PCR assay for the detection of Campylobacter in marinated and non-marinated poultry products." Food Microbiology **25**(7): 908-914.

Kazwala, R. R., J. D. Collins, et al. (1990). "Factors responsible for the introduction and spread of Campylobacter jejuni infection in commercial poultry production." Veterinary Record **126**(13): 305-306.

Keener, K. M., M. P. Bashor, et al. (2004). "Comprehensive Review of Campylobacter and Poultry Processing." Comprehensive Review in food science and food safety **3**(2): 105-116.

Kestin, S. C., T. G. Knowles, et al. "Prevalence of leg weakness in broiler chickens and its relationship with genotype." Veterinary Record **131**(9): 190-194.

Kestin, S. C., T. G. Knowles, et al. (1992). "Prevalence of leg weakness in broiler chickens and its relationship with genotype." Veterinary Record **191**(9): 190-194.

Keum-Il, J., K. Min-Gon, et al. (2007). "Morphology and adhesion of Campylobacter jejuni to chicken skin under varying conditions." Journal of Microbiology & Biotechnology **17**(2): 202-206.

Kiepper, B. (2001). "A survey of wastewater treatment practices in the broiler industry " Proceedings of the Water Environment Federation **50**: 12-25.

King, E. O. (1957). "Human infections with Vibrio fetus and a closely related vibrio." Journal of Infectious Diseases **101**(2): 119-128.

King, E. O. (1962). "The laboratory recognition of vibro fetus and a closely related vibrio isolated from cases oh human vibriosis." Annals of the New York Academy of Sciences **Volume 98 Laboratory Diagnosis of Microbial Diseases**: 700-711.

Kirkwood, B. R. and J. A. C. Sterne (2003). "Essential Medical Statistics."

Kittl, S., P. Kuhnert, et al. (2011). "Comparison of genotypes and antibiotic resistance of Campylobacter jejuni isolated from humans and slaughtered chickens in Switzerland." Journal of Applied Microbiology **110**(2): 513-520.

Klein, G., L. Beckmann, et al. (2007). "Predominant strains of thermophilic Campylobacter spp. in a German poultry slaughterhouse." International Journal of Food Microbiology **117**(3): 324-328.

Klein, G., F. Reich, et al. (2007). "Quantification of thermophilic Campylobacter spp. in broilers during meat processing." Antonie van Leeuwenhoek **92**(3): 267-273.

Klena, J. D., C. T. Parker, et al. (2004). "Differentiation of Campylobacter coli, Campylobacter jejuni, Campylobacter lari, and Campylobacter upsaliensis by a multiplex PCR developed from the nucleotide sequence of the lipid A gene lpxA." Journal of Clinical Microbiology **42**(12): 5549-5557.

Knowles, T. G. and D. M. Broom (1990). "The handling and transport of broilers and spent hens." Applied Animal Behaviour Science **28**(1): 79-91.

Knowles, T. G., S. C. Kestin, et al. (2008). "Leg disorders in broiler chickens: prevalence, risk factors and prevention." PLoS ONE [Electronic Resource] **3**(2): e1545.

Knudsen, K. N., D. D. Bang, et al. (2006). "Campylobacter jejuni strains of human and chicken origin are invasive in chickens after oral challenge." Avian Diseases **50**(1): 10-14.

Kovats, R. S., S. J. Edwards, et al. (2005). "Climate variability and campylobacter infection: an international study." International Journal of Biometeorology **49**(4): 207-214.

Kramer, J. M., J. A. Frost, et al. (2000). "Campylobacter contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection." Journal of Food Protection **63**(12): 1654-1659.

Kuana, S., L. Santos, et al. (2008). "Occurrence and Characterization of Campylobacter in the Brazilian Production and Processing of Broilers." Avian Diseases **52**: 680-684.

Kudirkienėa, E., J. Bunevičienėa, et al. (2011). "Evidence of broiler meat contamination with post-disinfection strains of Campylobacter jejuni from slaughterhouse " International Journal of Food Microbiology **145**(1).

Kudra, L. L., J. G. Sebranek, et al. (2012). "Control of Campylobacter jejuni in chicken breast meat by irradiation combined with modified atmosphere packaging including carbon monoxide." Journal of Food Protection **75**(10): 1728-1733.

Kusumaningrum, H. D., G. Riboldi, et al. (2003). "Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods." International Journal of Food Microbiology **85**(3): 227-236.

Kwan, P. S., A. Birtles, et al. (2008). "Longitudinal study of the molecular epidemiology of Campylobacter jejuni in cattle on dairy farms." Applied & Environmental Microbiology **74**(12): 3626-3633.

Kyvsgaard, N. C., H. B. Jensen, et al. (2013). "Temporal changes and risk factors for foot-pad dermatitis in Danish broilers." Poultry Science **92**(1): 26-32.

Lee, A., J. O'Rourke, et al. (1986). "Mucus colonization as a determinant of pathogenicity in intestinal infection by Campylobacter jejuni: a mouse cecal model." Infect Immun. **51 (2)**: 536-546.

Lee, A., S. C. Smith, et al. (1998). "Survival and growth of Campylobacter jejuni after artificial inoculation onto chicken skin as a function of temperature and packaging conditions." Journal of Food Protection **61**(12): 1609-1614.

Lehner, A., T. Tasara, et al. (2005). "Relevant aspects of Arcobacter spp. as potential foodborne pathogen." International Journal of Food Microbiology **102**(2): 127-135.

Leitner, G. and E. D. Heller (1992). "Colonization of Escherichia coli in young turkeys and chickens." Avian Diseases **36**(2): 211-220.

Lindblad, M., I. Hansson, et al. (2006). "Postchill campylobacter prevalence on broiler carcasses in relation to slaughter group colonization level and chilling system." Journal of Food Protection **69**(3): 495-499.

Lindqvist, R. and M. Lindblad (2008). "Quantitative risk assessment of thermophilic Campylobacter spp. and cross-contamination during handling of raw broiler chickens evaluating strategies at the producer level to reduce human campylobacteriosis in Sweden." International Journal of Food Microbiology **121**(1): 41-52.

Linton, D., R. J. Owen, et al. (1996). "Rapid identification by PCR of the genus Campylobacter and of five Campylobacter species enteropathogenic for man and animals." Research in Microbiology **147**(9): 707-718.

Little, C. L., J. F. Richardson, et al. (2008). "Campylobacter and Salmonella in raw red meats in the United Kingdom: prevalence, characterization and antimicrobial resistance pattern, 2003-2005." Food Microbiology **25**(3): 538-543.

Loretz, M., R. Stephan, et al. (2010). "Antimicrobial activity of decontamination treatments for poultry carcasses: A literature survey." Food Control **21**(6): 791-804.

Louis, V., I. Gillespie, et al. (2005). "Temperature-driven Campylobacter seasonality in England and Wales." Applied & Environmental Microbiology **71**(1): 85-92.

Luangtongkum, T., T. Y. Morishita, et al. (2006). "Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of Campylobacter spp. in poultry." Applied & Environmental Microbiology **72**(5): 3600-3607.

Luber, P. and E. Bartelt (2007). "Enumeration of Campylobacter spp. on the surface and within chicken breast fillets." Journal of Applied Microbiology **102**(2): 313-318.

Luber, P., S. Brynestad, et al. (2006). "Quantification of campylobacter species cross-contamination during handling of contaminated fresh chicken parts in kitchens." Applied & Environmental Microbiology **72**(1): 66-70.

Lyte, M. and S. Ernst (1992). "Catecholamine induced growth of gram negative bacteria." Life Sciences **50**(3): 203-212.

Maiden, M. C., J. A. Bygraves, et al. (1998). "Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms." Proceedings of the National Academy of Sciences of the United States of America **95**(6): 3140-3145.

Manning, G., C. G. Dowson, et al. (2003). "Multilocus sequence typing for comparison of veterinary and human isolates of Campylobacter jejuni." Applied & Environmental Microbiology **69**(11): 6370-6379.

Martland, M. F. (1985). "Ulcerative dermatitis in broiler chickens: the effects of wet litter." Avian Pathology **14**(3): 353-364.

Martrenchar, A., E. Boilletot, et al. (2002). "Risk factors for foot-pad dermatitis in chicken and turkey broilers in France." Preventive Veterinary Medicine **52**(3-4): 213-226.

Mashaly, M. M., G. L. Hendricks, 3rd, et al. (2004). "Effect of heat stress on production parameters and immune responses of commercial laying hens." Poultry Science **83**(6): 889-894.

Mason, M. J., T. J. Humphrey, et al. (1999). "Isolation of sublethally injured campylobacters from poultry and water sources." British Journal of Biomedical Science **56**(1): 2-5.

Mayne, R. K., R. W. Else, et al. (2007). "High litter moisture alone is sufficient to cause footpad dermatitis in growing turkeys." British Poultry Science **48**(5): 538-545.

McDowell, S. W., F. D. Menzies, et al. (2008). "Campylobacter spp. in conventional broiler flocks in Northern Ireland: epidemiology and risk factors." Preventive Veterinary Medicine **15**(84): 261-276.

McGeown, D., T. C. Danbury, et al. (1999). "Effect of carprofen on lameness in broiler chickens." Veterinary Record **144**(24): 668-671.

McNair, D., C. Spelman, et al. (2013). "The Mcnair report. An independent inquiry and report commissioned by the RSPCA into the Freedom Food animal welfare assurance scheme."

Mead, C. (2002). "Factors affecting intestinal colonisation of poultry by campylobacter and role of microflora in control." World Poultry Science JournalFactors affecting intestinal

colonisation of poultry by campylobacter and role of microflora in control **58**: 169–178.

Mead, G. C., W. R. Hudson, et al. (1995). "Effect of changes in processing to improve hygiene control on contamination of poultry carcasses with campylobacter." Epidemiology & Infection **115**(3): 495-500.

Meldrum, R. J. and I. G. Wilson (2007). "Salmonella and Campylobacter in United Kingdom retail raw chicken in 2005." Journal of Food Protection **70**(8): 1937-1939.

Mench, J. A. (1991). "Research note: feed restriction in broiler breeders causes a persistent elevation in corticosterone secretion that is modulated by dietary tryptophan." Poulty Science **70**(12): 2547-2550.

Messens, W., L. Herman, et al. (2009). "Multiple typing for the epidemiological study of contamination of broilers with thermotolerant Campylobacter." Veterinary Microbiology **138**(1-2): 120-131.

Methner, U., W. Rabsch, et al. (2008). "Effect of norepinephrine on colonisation and systemic spread of Salmonella enterica in infected animals: role of catecholate siderophore precursors and degradation products." International Journal of Medical Microbiology **298**(5-6): 429-439.

Meyer, A., T. Stallmach, et al. (1997). "Lethal maternal sepsis caused by Campylobacter jejuni: pathogen preserved in placenta and identified by molecular methods." Modern Pathology **10**(12): 1253-1256.

Miller, W. G., S. L. On, et al. (2005). "Extended multilocus sequence typing system for Campylobacter coli, C. lari, C. upsaliensis, and C. helveticus." Journal of Clinical Microbiology **43**(5): 2315-2329.

Mitchella, M. A. and P. J. Kettlewella (1994). "Road transportation of broiler chickens: induction of physiological stress." World's Poultry Science Journal **50**(1): 57-59.

Miwa, N., Y. Takegahara, et al. (2003). "Campylobacter jejuni contamination on broiler carcasses of C. jejuni-negative flocks during processing in a Japanese slaughterhouse." International Journal of Food Microbiology **84**(1): 105-109.

Moore, J. E., D. Corcoran, et al. (2005). "Campylobacter." Veterinary Research **36**(3): 351-382.

Mulder, M. W. A. W. (1995). "Impact of transport and related stresses on the incidence and extent of human pathogens in pigmeat and poultry." Journal of Food Safety **15**(3): 239-246.

Mullner, P., J. M. Collins-Emerson, et al. (2010). "Molecular epidemiology of Campylobacter jejuni in a geographically isolated country with a uniquely structured poultry industry." Applied & Environmental Microbiology **76**(7): 2145-2154.

Mullner, P., T. Shadbolt, et al. (2010). "Molecular and spatial epidemiology of human campylobacteriosis: source association and genotype-related risk factors." Epidemiology & Infection **138**(10): 1372-1383.

Mullner, P., S. E. Spencer, et al. (2009). "Assigning the source of human campylobacteriosis in New Zealand: a comparative genetic and epidemiological approach." Infection, Genetics & Evolution **9**(6): 1311-1319.

Murphy, C., C. Carroll, et al. (2006). "Environmental survival mechanisms of the foodborne pathogen Campylobacter jejuni." Journal of Applied Microbiology **100**(4): 623-632.

Murphy, D., C. Jolly, et al. (2013). "Myocarditis associated with Campylobacter jejuni." New Zealand Medical Journal **126**(1375): 95-98.

Musgrove, M. T., M. E. Berrang, et al. (2001). "Detection of Campylobacter spp. in ceca and crops with and without enrichment." Poultry Science **80**(6): 825-828.

Musgrove, M. T., J. A. Cason, et al. (1997). "Effect of cloacal plugging on microbial recovery from partially processed broilers." Poultry Science **76**(3): 530-533.

Nachamkin, I., B. Allos, et al. (1998). "Campylobacter Species and Guillain-Barré Syndrome." Clinical Microbiology Reviews **11**: 555-567.

Nauta, M., A. Hill, et al. (2009). "A comparison of risk assessments on Campylobacter in broiler meat." International Journal of Food Microbiology **129**(2): 107-123.

Nauta, M., I. van der Fels-Klerx, et al. (2005). "A poultry-processing model for quantitative microbiological risk assessment." Risk Analysis **25**(1): 85-98.

Nauta, M. J., W. F. Jacobs-Reitsma, et al. (2007). "A risk assessment model for Campylobacter in broiler meat." Risk Analysis **27**(4): 845-861.

Neimann, J., J. Engberg, et al. (2003). "A case-control study of risk factors for sporadic campylobacter infections in Denmark." Epidemiology & Infection **130**(3): 353-366.

Nereus, W. G. and C.-Y. Chen (2009). "The biofilm forming potential of bacterial species in the genus Campylobacter." Food Microbiology **26**(44-51): 44-51.

Newell, D. G., K. T. Elvers, et al. (2011). "Biosecurity-Based Interventions and Strategies To Reduce Campylobacter spp. on Poultry Farms." Applied Environmental Microbiology **77**(24).

Newell, D. G. and C. Fearnley (2003). "Sources of Campylobacter colonization in broiler chickens." Applied & Environmental Microbiology **69**(8): 4343-4351.

Newell, D. G., H. McBride, et al. (1985). "The virulence of clinical and environmental isolates of Campylobacter jejuni." Journal of Hygiene **94**(1): 45-54.

Newell, D. G., J. E. Shreeve, et al. (2001). "Changes in the carriage of Campylobacter strains by poultry carcasses during processing in abattoirs." Applied & Environmental Microbiology **67**(6): 2636-2640.

Ng, L. K., R. Sherburne, et al. (1985). "Morphological forms and viability of Campylobacter species studied by electron microscopy." Journal of Bacteriology **164**(1): 338-343.

Nguyen, V. T., N. Fegan, et al. (2012). "Role of attachment to surfaces on the prevalence and survival of Campylobacter through food systems." Journal of Food Protection **75**(1): 195-206.

Nguyen, V. T., M. S. Turner, et al. (2011). "Influence of cell surface hydrophobicity on attachment of Campylobacter to abiotic surfaces." Food Microbiology **28**(5): 942-950.

Nichols, G. and L. Gordon (2005). "Fly transmission of Campylobacter." Emerging Infectious Diseases **11**(3): 361-364.

Nichols, G. L., J. F. Richardson, et al. (2012). "Campylobacter epidemiology: a descriptive study reviewing 1 million cases in England and Wales between 1989 and 2011." BMJ Open **2**(4).

Nicol, C. J. and G. B. Scott (1990). "Pre-slaughter handling and transport of broiler chickens." Applied Animal Behaviour Science **28**(1): 57-73.

Niederer, L., P. Kuhnert, et al. (2012). "Genotypes and antibiotic resistances of Campylobacter jejuni and Campylobacter coli isolates from domestic and travel-associated human cases." Applied & Environmental Microbiology **78**(1): 288-291.

Normand, V., M. Boulianne, et al. (2008). "Evidence of cross-contamination by Campylobacter spp. of broiler carcasses using genetic characterization of isolates." Canadian Journal of Veterinary Research **72**(5): 396-402.

Northcutt, J. K., M. E. Berrang, et al. (2003). "Effect of broiler age, feed withdrawal, and transportation on levels of coliforms, Campylobacter, Escherichia coli and Salmonella on carcasses before and after immersion chilling." Poultry Science **82**(1): 169-173.

O'Brien, S. J. (2013). "The "Decline and Fall" of Nontyphoidal Salmonella in the United Kingdom." Clinical Infectious Diseases **56**(5): 705-710.

OIE (2009). "OIE Guidelines for the on-farm welfare of chickens raised for meat ".

Oliver, J. D. (2005). "The viable but nonculturable state in bacteria." Journal of Microbiology **43 Spec No**: 93-100.

Olson, C. K., S. Ethelberg, et al. (2008). "Epidemiology of Campylobacter jejuni infections in industrialized nations." Campylobacter 163-189.

Padungton, P. and J. B. Kaneene (2003). "Campylobacter spp in human, chickens, pigs and their antimicrobial resistance." Journal of Veterinary Medical Science **65**(2): 161-170.

Park, S. F. (2002). "The physiology of Campylobacter species and its relevance to their role as foodborne pathogens." International Journal of Food Microbiology **74**(3): 177-188.

Patriarchi, A., A. Fox, et al. (2011). "Molecular characterization and environmental mapping of Campylobacter isolates in a subset of intensive poultry flocks in Ireland." Foodborne Pathogens & Disease **8**(1): 99-108.

Pearson, A. D., M. Greenwood, et al. (1993). "Colonization of broiler chickens by waterborne Campylobacter jejuni." Applied & Environmental Microbiology **59**(4): 987-996.

Pebody, R. G., M. J. Ryan, et al. (1997). "Outbreaks of campylobacter infection: rare events for a common pathogen." Communicable Disease Report. CDR Review **7**(3): R33-37.

Perko-Mäkelä, P., M. Hakkinen, et al. (2002). "Prevalence of campylobacters in chicken flocks during the summer of 1999 in Finland." Epidemiology and Infection **129**(1): 187-192.

Peterson, P. K., C. C. Chao, et al. (1991). "Stress and pathogenesis of infectious disease." Reviews of Infectious Diseases **13**(4): 710-720.

Peyrat, M. B., C. Soumet, et al. (2008). "Phenotypes and genotypes of campylobacter strains isolated after cleaning and disinfection in poultry slaughterhouses." Veterinary Microbiology **128**(3-4): 313-326.

Peyrat, M. B., C. Soumet, et al. (2008). "Recovery of Campylobacter jejuni from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination." International Journal of Food Microbiology **124**(2): 188-194.

Pointon, A., M. Sexton, et al. (2008). "A baseline survey of the microbiological quality of chicken portions and carcasses at retail in two Australian states (2005 to 2006)." Journal of Food Protection **71**(6): 1123-1134.

Pope, J. E., A. Krizova, et al. (2007). "Campylobacter reactive arthritis: a systematic review." Seminars in Arthritis & Rheumatism **37**(1): 48-55.

Ragimbeau, C., F. Schneider, et al. (2008). "Multilocus sequence typing, pulsed-field gel electrophoresis, and fla short variable region typing of clonal complexes of Campylobacter jejuni strains of human, bovine, and poultry origins in Luxembourg." Applied & Environmental Microbiology **74**(24): 7715-7722.

Rahimi, E., H. Kazemeini, et al. (2010). "Detection and identification of Campylobacter spp. from retail raw chicken, turkey, sheep and goat meat in Ahvaz, Iran." African journal of microbiology research **4**: 1620-1623.

Ramabu, S. S., N. S. Boxall, et al. (2004). "Some potential sources for transmission of Campylobacter jejuni to broiler chickens." Letters in Applied Microbiology **39**(3): 252-256.

Rao, M. R., A. B. Naficy, et al. (2001). "Pathogenicity and convalescent excretion of Campylobacter in rural Egyptian children." American Journal of Epidemiology **154**(2): 166-173.

Rasschaert, G., K. Houf, et al. (2006). "Campylobacter contamination during poultry slaughter in Belgium." Journal of Food Protection **69**(1): 27-33.

Rauwa, W. M., E. Kanisb, et al. (1998). "Undesirable side effects of selection for high production efficiency in farm animals: a review." Livestock Production Science **56**(1): 15-33.

Reeser, R. J., R. T. Medler, et al. (2007). "Characterization of Campylobacter jejuni biofilms under defined growth conditions." Applied & Environmental Microbiology **73**(6): 1908-1913.

Reich, F., V. Atanassova, et al. (2008). "The effects of Campylobacter numbers in caeca on the contamination of broiler carcasses with Campylobacter." International Journal of Food Microbiology **127**(1-2): 116-120.

Richardson, L. J., N. A. Cox, et al. (2011). "Isolation of Campylobacter from circulating blood of commercial broilers." Avian Diseases **55**(3): 375-378.

Ridley, A. M., V. K. Morris, et al. (2011). "Longitudinal molecular epidemiological study of thermophilic campylobacters on one conventional broiler chicken farm." Applied & Environmental Microbiology **77**(1): 98-107.

Rivoal, K., M. Denis, et al. (1999). "Molecular characterization of the diversity of Campylobacter spp. isolates collected from a poultry slaughterhouse: analysis of cross-contamination." Letters in Applied Microbiology **29**(6): 370-374.

Roberts, J. A., P. Cumberland, et al. (2003). "The study of infectious intestinal disease in England: socio-economic impact." Epidemiology and Infection **130**(1): 1-11.

Robinson, D. A. (1981). "Infective dose of Campylobacter jejuni in milk." British Medical Journal **282**.

Rodgers, J. D., F. A. Clifton-Hadley, et al. (2010). "An evaluation of survival and detection of Campylobacter jejuni and C. coli in broiler caecal contents using culture-based methods." Journal of Applied Microbiology **109**(4): 1244-1252.

Rollins, D. M. and R. R. Colwell (1986). "Viable but nonculturable stage of Campylobacter jejuni and its role in survival in the natural aquatic environment." Applied & Environmental Microbiology **52**(3): 531-538.

Rosales, G. (1994). "Managing stress in broiler breederas: a review." Applied Poultry Research **2**: 199-207.

Rosenquist, H., N. L. Nielsen, et al. (2003). "Quantitative risk assessment of human campylobacteriosis associated with thermophilic Campylobacter species in chickens." International Journal of Food Microbiology **83**(1): 87-103.

Rosenquist, H., H. M. Sommer, et al. (2006). "The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant Campylobacter." International Journal of Food Microbiology **108**(2): 226-232.

Rostagno, M. (2009). "Can stress in farm animals increase food safety risk?" Foodborne Pathog Dis. **6**(7): 767-776.

RSPCA (2011). "RSPCA welfare standards for chickens."

RTFA (2011). "Red Tractor Farm Assurance Poultry Scheme (formerly ACP)."

Rushton, S. P., T. J. Humphrey, et al. (2009). "Campylobacter in housed broiler chickens: a longitudinal study of risk factors." Epidemiology & Infection **137**(8): 1099-1110.

Sahin, O., T. Morishita, et al. (2002). "Campylobacter colonization in poultry: sources of infection and modes of transmission." Animal Health Research Reviews **3**(2): 95-105.

Sahin, O., L. Naidan, et al. (2003). "Effect of Campylobacter-Specific Maternal Antibodies on Campylobacter jejuni Colonization in Young Chickens " Applied and Environmental Microbiology **69**(9): 5372-5379.

Sahin, O., Q. Zhang, et al. (2001). "Prevalence, antigenic specificity, and bactericidal activity of poultry anti-Campylobacter maternal antibodies." Applied & Environmental Microbiology **67**(9): 3951-3957.

Sails, A. D., B. Swaminathan, et al. (2003). "Utility of multilocus sequence typing as an epidemiological tool for investigation of outbreaks of gastroenteritis caused by Campylobacter jejuni." Journal of Clinical Microbiology **41**(10): 4733-4739.

Saleha, A. A. (2004). "Epidemiological Study on the Colonization of Chickens with Campylobacter in Broiler Farms in Malaysia : Possible Risk and Management Factors." International Journal of Poultry Science **2**(2): 129-134.

Sampers, I., I. Habib, et al. (2008). "Processing practices contributing to Campylobacter contamination in Belgian chicken meat preparations." International Journal of Food Microbiology **128**(2): 297-303.

Sánchez, M. X., W. M. Fluckey, et al. (2002). "Microbial profile and antibiotic susceptibility of Campylobacter spp. and Salmonella spp. in broilers processed in air-chilled and immersion-chilled environments." Journal of food protection **65**(6): 948-956.

Sandberg, M., M. Hofshagen, et al. (2005). "Survival of Campylobacter on frozen broiler carcasses as a function of time." Journal of Food Protection **68**(8): 1600-1605.

Sanotraa, G. S., J. D. Lunda, et al. (2001). "Monitoring leg problems in broilers: a survey of commercial broiler production in Denmark." World's Poultry Science Journal **57**(01): 55-69.

Sanyal, S. C., K. M. Islam, et al. (1983). "Campylobacter jejuni diarrhea model in infant chickens." Infection & Immunity **43**(3): 931-936.

Scientific Committee on Animal Health and Animal Welfare (2000). "The Welfare of Chickens Kept for Meat Production (Broilers)."

SCVPH (1998). "Benefits and limitation of antimicrobial treatments for poutry carcasses."

Sebald, M. and M. Veron (1963). "Teneur en bases de l' ADN et classification des vibrions." Ann Inst Pasteur (Paris) 1963 Nov;105:897–910 **105**: 897–910.

Shane, S. M. (2000). "Campylobacter infection of commercial poultry." Revue Scientifique et Technique **19**(2): 376-395.

Shanker, S., A. Lee, et al. (1986). "Campylobacter jejuni in broilers: the role of vertical transmission." Journal of Hygiene **96**(2): 153-159.

Shanker, S., A. Lee, et al. (1990). "Horizontal transmission of Campylobacter jejuni amongst broiler chicks: experimental studies." Epidemiology & Infection **104**(1): 101-110.

Shepherd, E. M. and B. D. Fairchild (2010). "Footpad dermatitis in poultry." Poultry Science **89**(10): 2043-2051.

Sheppard, A. (2004). "The Structure and Economics of Broiler Production in England."

Sheppard, S. K., J. F. Dallas, et al. (2009). "Campylobacter genotyping to determine the source of human infection." Clinical Infectious Diseases **48**(8): 1072-1078.

Shini, S., G. R. Huff, et al. (2010). "Understanding stress-induced immunosuppression: exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes." Poultry Science **89**(4): 841-851.

Shreeve, J. E., M. Toszeghy, et al. (2000). "Sequential spread of Campylobacter infection in a multipen broiler house." Avian Diseases **44**(4): 983-988.

Shreeve, J. E., M. Toszeghy, et al. (2002). "The carry-over of Campylobacter isolates between sequential poultry flocks." Avian Diseases **46**(2): 378-385.

Silva, J., D. Leite, et al. (2011). "Campylobacter spp. as a Foodborne Pathogen: A Review." Frontiers in microbiology **2**(200).

Silva, R., I. Nääs, et al. (2011). "Poultry welfare scenario in South America: norms and regulations." Brazilian Journal of Poultry Science **13** (2): 83-89.

Simpson, E. H. (1494). "Measurement of Diversity." Nature **163**: 688-.

Skirrow, M. B. (1977). "Campylobacter enteritis: a "new" disease." British Medical Journal **2**(6078): 9-11.

Skirrow, M. B. (2006). "John McFadyean and the centenary of the first isolation of Campylobacter species." Clinical Infectious Diseases **43**(9): 1213-1217.

Slader, J., G. Domingue, et al. (2002). "Impact of transport crate reuse and of catching and processing on Campylobacter and Salmonella contamination of broiler chickens." Applied & Environmental Microbiology **68**(2): 713-719.

Smith, T. and M. Taylor (1919). "Some morphological and biological characters of the spirilla (Vibro Fetus) associated with disease of the fetal membranes in cattle." J Exp Med. **30(4)**: 299-311.

Solomon, E. B. and D. G. Hoover (1999). "Campylobacter Jejuni: a bacterial paradox." Journal of Food Safety **19**: 121-136.

Son, I., M. D. Englen, et al. (2007). "Prevalence of Arcobacter and Campylobacter on broiler carcasses during processing." International Journal of Food Microbiology **113**(1): 16-22.

Stahl, M., J. Butcher, et al. (2012). "Nutrient acquisition and metabolism by Campylobacter jejuni." Frontiers in Cellular and Infection Microbiology **2**(5).

Stahl, M., L. M. Friis, et al. (2011). "L-fucose utilization provides Campylobacter jejuni with a competitive advantage." Proceedings of the National Academy of Sciences of the United States of America **108**(17): 7194-7199.

StataCorp (2005). "Stata Data Analysis and Statistical Software: Release 9."

Stephens, C. P., S. L. On, et al. (1998). "An outbreak of infectious hepatitis in commercially reared ostriches associated with Campylobacter coli and Campylobacter jejuni." Veterinary Microbiology **61**(3): 183-190.

Stern, N. J., M. R. Clavero, et al. (1995). "Campylobacter spp. in broilers on the farm and after transport." Poultry Science **74**(6): 937-941.

Stern, N. J., P. Fedorka-Cray, et al. (2001). "Distribution of Campylobacter spp. in Selected U.S. Poultry Production and Processing Operations." Journal of Food Protection **64**(11): 1705-1710.

Stern, N. J., K. L. Hiett, et al. (2003). "Campylobacter spp. in Icelandic poultry operations and human disease." Epidemiology & Infection **130**(1): 23-32.

Stern, N. J., R. J. Meinersmann, et al. (1990). "Influence of host lineage on cecal colonization by Campylobacter jejuni in chickens." Avian Diseases **34**(3): 602-606.

Stern, N. J., J. Reiersen, et al. (2005). "Occurrence of Campylobacter spp. in cecal contents among commercial broilers in iceland." Foodborne Pathogens & Disease **2**(1): 82-89.

Stern, N. J., E. A. Svetoch, et al. (2005). "Paenibacillus polymyxa purified bacteriocin to control Campylobacter jejuni in chickens." Journal of Food Protection **68**(7): 1450-1453.

Stern, N. J. B., L. Blankenship, N. Cox and F. McHan (1988). "Colonization characteristics of Campylobacter jejuni in chick ceca." Avian Dis. **32**: pp. 330–334.

Swart, A. N., M. J. J. Mangen, et al. (2013). "Microbiological criteria as a decision tool for controlling Campylobacter in the broiler meat chain."

Tam, C. C., L. C. Rodrigues, et al. (2003). "Guillain-Barre syndrome associated with Campylobacter jejuni infection in England, 2000-2001." Clinical Infectious Diseases **37**(2): 307-310.

Tam, C. C., L. C. Rodrigues, et al. (2006). "Temperature dependence of reported Campylobacter infection in England, 1989-1999." Epidemiology & Infection **134**(1): 119-125.

Tam, C. C., L. C. Rodrigues, et al. (2006). "Incidence of Guillain-Barré syndrome among patients with Campylobacter infection: a general practice research database study." Journal of Infectious Diseases **194**(1).

Tam, C. C., L. C. Rodrigues, et al. (2012). "Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice." Gut **61**(1): 69-77.

Taveirne, M. E., C. M. Theriot, et al. (2013). "The Complete Campylobacter jejuni Transcriptome during Colonization of a Natural Host Determined by RNAseq." PLoS ONE [Electronic Resource] **8**(8).

Tustin, J., Laberge, K., Michel, P., Reiersen, J., Dađadóttir, S., Briem, H., Harđardóttir, H., Kristinsson, K., Gunnarsson, E., Friđriksdóttir, V. and Georgsson, F. (2010). "A National Epidemic of Campylobacteriosis in Iceland, Lessons Learned." Zoonoses and Public Health **58**.

USDA (2011). "EU-27, Poultry and Products Semi-annual, EU-27 Poultry Sector Growth Slowing in 2011."

Valkenburgh, S., R. , O. S. van Oosterom, et al. (2007). "Zoonoses and Zoonotic Agents in Humans, Food, Animals and Feed in the Netherlands 2003-2006."

van de Giessen, A., S. I. Mazurier, et al. (1992). "Study on the epidemiology and control of Campylobacter jejuni in poultry broiler flocks." Applied & Environmental Microbiology **58**(6): 1913-1917.

van Der Wielen, P. W., S. Biesterveld, et al. (2000). "Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth." Applied & Environmental Microbiology **66**(6): 2536-2540.

Van Deun, K., F. Haesebrouck, et al. (2007). "Virulence properties of Campylobacter jejuni isolates of poultry and human origin." Journal of Medical Microbiology **56**(Pt 10): 1284-1289.

Van Gerwe, T. J. W. M., A. Bouma, et al. (2005). "Quantifying transmission of Campylobacter spp. among broilers." Applied & Environmental Microbiology **71**(10): 5765-5770.

Van Horne, P. I. and T. J. Achterbosch (2008). "Animal welfare in poultry production system: impact of EU standards on world trade." World`s Poultry Science Journal **64**.

Vandamme, P. and J. De Ley (1991). "Proposal for a New Family, Campylobacteraceae." International Journal of systematic bacteriology: 451-455.

Vandeplas, S. and R. Dubois-Dauphin (2010). "Prevalence and sources of Campylobacter spp. contamination in free-range broiler production in the southern part of Belgium." Biotechnol. Agron. Soc. Environ. **14**(2): 279-288.

Vandeplas, S., R. Dubois-Dauphin, et al. (2010). "Prevalence and sources of Campylobacter spp. contamination in free-range broiler production in the southern part of Belgium." Biotechnologie, Agronomie, Société et Environnement **14**(2): 279-288.

Vandeplas, S., D. R. Dubois, et al. (2009). "Response of the specific immune system on cecal colonization of slow-growing broiler chickens reared on litter contaminated by Campylobacter jejuni."

Vellinga, A. and F. Van Loock (2002). "The dioxin crisis as experiment to determine poultry-related campylobacter enteritis." Emerging Infectious Diseases **8**(1): 19-22.

Veron, M. and R. Chatelain (1973). "Taxonomic Study of the Genus Campylobacter Sebald and Vkron and Designation of the Neotype Strain for the Type Species, Campylobacter fetus (Smith and Taylor) Sebald and Vkon." International Journal of systematic bacteriology

122-134.

Viator, C. L., S. C. Cates, et al. (2008). "Adoption of interventions to improve food safety at meat and poultry processing plants in the United States." Food Protection Trends **28**: 917-927.

Wabeck, C. J. (1972). "Feed and Water Withdrawal Time Relationship to Processing Yield and Potential Fecal Contamination of Broilers." Poultry Science **51**(4): 1119-1121.

Wagenaar, J. A., D. J. Mevius, et al. (2006). "Campylobacter in primary animal production and control strategies to reduce the burden of human campylobacteriosis." Revue Scientifique et Technique **25**(2): 581-594.

Wagenaar, J. A., M. A. P. Van Bergen, et al. (2005). "Phage therapy reduces Campylobacter jejuni colonization in broilers." Veterinary Microbiology **109**(3-4): 275-283.

Walker, R. I., M. B. Caldwell, et al. (1986). "Pathophysiology of Campyfobacter species." Journal of Bacteriology **72**: 949-955.

Wallace, J. S., K. N. Stanley, et al. (1997). "Seasonality of thermophilic Campylobacter populations in chickens." Journal of Applied Microbiology **82**(2): 219-224.

Walsh, P. S., D. A. Metzger, et al. (1991). "Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material." Biotechniques **10**(4): 506-513.

Wassenaar, T. M. and D. G. Newell (2000). "Genotyping of Campylobacter spp." Applied & Environmental Microbiology **66**(1): 1-9.

Wempe, J. M., C. A. Genigeorgis, et al. (1983). "Prevalence of Campylobacter jejuni in two California chicken processing plants." Applied & Environmental Microbiology **45**(2): 355-359.

WHO (2000). "The Increasing Incidence of Human Campylobacteriosis. Report and Proccedings of a WHO Consultation of Experts."

Whyte, P., J. D. Collins, et al. (2001). "Distribution and prevalence of airborne microorganisms in three commercial poultry processing plants." Journal of Food Protection **64**(3): 388-391.

Whyte, P., J. D. Collins, et al. (2001). "The effect of transportation stress on excretion rates of campylobacters in market-age broilers." Poultry Science **80**(6): 817-820.

Wieliczko, A. (1994). "Occurrence of Campylobacter and salmonellas in relation to liver changes in slaughtered poultry." Berliner und Munchener Tierarztliche Wochenschrift **107**(4): 115-121.

Williams, L. K., A. Sait, et al. (2013). "Campylobacter Infection Has Different Outcomes in Fast- and Slow-Growing Broiler Chickens." Avian Diseases **57**(238-241).

Williams, L. K., L. C. Sait, et al. (2012). "Enrichment culture can bias the isolation of Campylobacter subtypes." Epidemiology & Infection **140**(7): 1227-1235.

Wilson, D. J., E. Gabriel, et al. (2008). "Tracing the source of campylobacteriosis." PLoS Genetics **4**(9): e1000203.

Wilson, D. J., E. Gabriel, et al. (2008). "Tracing the source of campylobacteriosis." PLoS Genetics **4**(9): e1000203.

Wingstrand, A., J. Neimann, et al. (2006). "Fresh chicken as main risk factor for campylobacteriosis, Denmark." Emerging Infectious Diseases **12**(2): 280-285.

Wirz, S. E., G. Overesch, et al. (2010). "Genotype and antibiotic resistance analyses of Campylobacter isolates from ceca and carcasses of slaughtered broiler flocks." Applied & Environmental Microbiology **76**(19): 6377-6386.

Yang, H., Y. Li, et al. (2001). "Survival and death of Salmonella typhimurium and Campylobacter jejuni in processing water and on chicken skin during poultry scalding and chilling." Journal of Food Protection **64**(6): 770-776.

Ziprin, R. L., R. E. Droleskey, et al. (2003). "Failure of viable nonculturable Campylobacter jejuni to colonize the cecum of newly hatched leghorn chicks." Avian Diseases **47**(3): 753-758.

Zweifel, C., K. D. Scheu, et al. (2008). "Occurrence and genotypes of Campylobacter in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms." International Journal of Food Microbiology **125**(2): 182-187.